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THE EFFECT OF INORGANIC PARTICLES ON METABOLISM BY MARINE BACTE--ETC(U)
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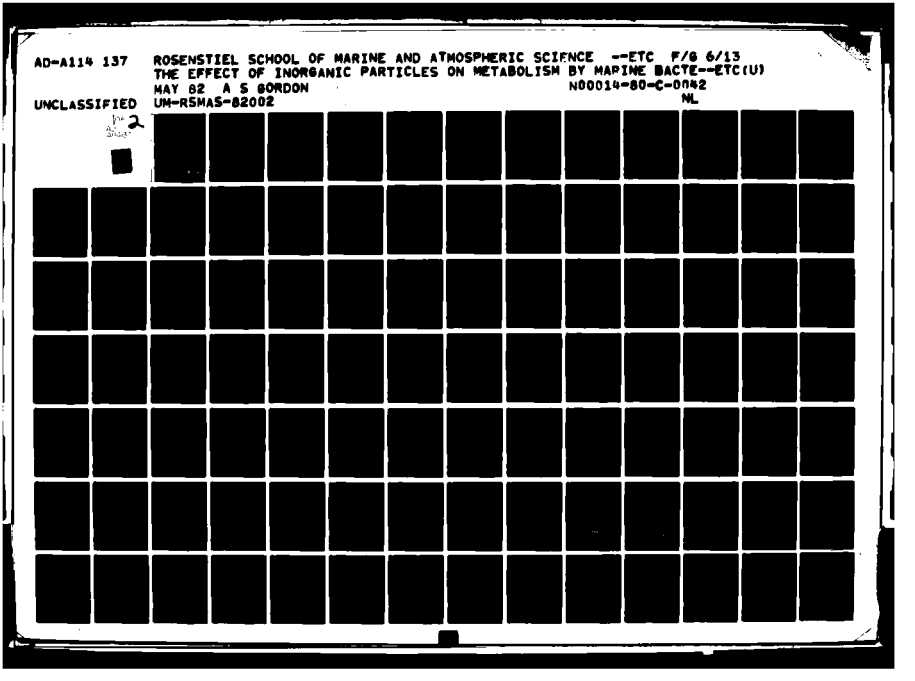

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UNIVERSITY OF MIAMI
ROSENSTIEL SCHOOL OF MARINE AND ATMOSPHERIC SCIENCE
4600 Rickenbacker Causeway
Miami, Florida 33149

TECHNICAL REPORT

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by

Andrew S. Gordon

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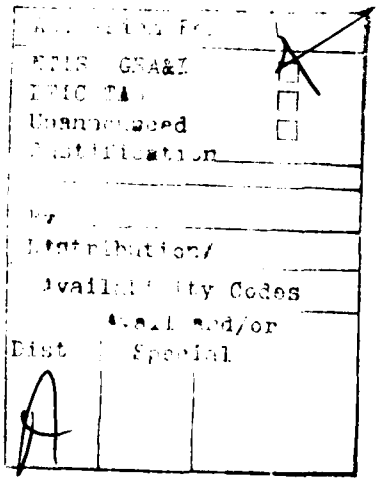
A Report to the Office of Naval Research
of Research Supported by
Contract N00014-80-C-0042

UM-RSMAS-82002



Warren J. Wisby
Interim Dean

GORDON, ANDREW SIRKOSKY (Ph.D., Microbiology)

The Effect of Inorganic Particles on Metabolism by Marine Bacteria. (May, 1982)

Abstract of a doctoral dissertation at the University of Miami. Dissertation supervised by Drs. S.M. Gerchakov and F.J. Millero.

The hypothesis tested in this study was that inorganic particles stimulate bacterial metabolism in dilute solutions due to concentration of organic nutrients and bacteria from solution by adsorption.

Measurements were made of adsorption of bacteria, glucose, and glutamic acid to inorganic particles in seawater and defined bacterial growth medium. Measurements of the metabolism of bacteria were made in the presence and absence of particles by microcalorimetry and radiorespirometry.

Hydroxyapatite adsorbs glutamic acid but not glucose from the experimental medium. Hydroxyapatite also adsorbs a significant fraction of bacterial cells from the medium if the bacterial concentration is below $6*10^5$ bacteria per milliliter. If bacterial concentrations are $6*10^7$, then only a small fraction of cells become attached. It was therefore possible to select bacterial concentrations and

organic nutrients so that bacterial attachment, organic nutrient adsorption, or both, would occur in different experiments. In this experimental system the metabolism by attached and non-attached bacteria of adsorbed and non-adsorbed organic nutrients was measured.

The results of the present study do not support the initial supposition that the activity of marine bacteria is stimulated by the presence of inorganic surfaces. Bacterial activity was not enhanced at high (millimolar) concentrations of glucose or at low concentrations (micromolar) of glucose or glutamic acid. Bacterial activity was not enhanced regardless of whether the bacteria, the organic nutrient or both were associated with the surface. In fact, the activity of the attached bacteria was diminished in comparison with free bacteria.

The results are discussed in terms of the possible reasons why bacteria attach to inorganic surfaces in aquatic environments.

ACKNOWLEDGEMENTS

I thank Drs. Sol M. Gerchakov and Frank J. Millero for guidance and encouragement during the course of this project. I also thank the other members of my committee for their criticisms and contributions.

The financial support for this project was received from the Office of Naval Research contracts N00014-80-C-0042 and N00014-76-C-0076.

I would also like to thank my family for their moral support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

This dissertation concerns heterotrophic marine bacteria and the manner in which inorganic solid surfaces can affect their metabolism. In particular the question asked in this study is: does adsorption of organic nutrients and/or bacteria onto the surfaces of inorganic particles stimulate the activity of these bacteria when the level of organic nutrient in solution is growth limiting. The motivation for this research is a hope of contributing to an understanding of why bacteria attach to inorganic surfaces in seawater.

Heterotrophic bacteria are those which derive their carbon and energy from organic nutrients (Davis et al., 1973). Nutrients are substances which organisms must draw from their environment for synthesis of cellular material and generation of energy (Stanier et al., 1976). The metabolic activity and growth of heterotrophic marine bacteria are, by definition, dependent on the availability of nutrients (Stanier et al., 1976). In natural water systems, nutrients are often limiting to the rate of growth and metabolism of microorganisms (Sieburth,1981). It is generally believed that readily metabolizable organic material is the nutrient which controls heterotrophic activity in the oceans

(Rheinheimer, 1974). Inorganic nutrients, especially sources of nitrogen and phosphorus, are also at low levels in some ocean waters (Riley and Chester, 1971) and may control the rate of heterotrophic activity there depending on competition between phototrophs and heterotrophs for these nutrients (Rheinheimer, 1974). An understanding of the factors controlling the rate of heterotrophic bacterial activity in aquatic ecosystems is necessary for determining the turnover rate of labile organic matter and its fate in natural waters. In addition, since bacteria serve as food for other organisms (Seiburth, 1981; Seki, 1972; Pomeroy, 1980), the growth rate and production of biomass by bacteria in aquatic systems is an important factor for determining the extent of their role in ecosystem productivity.

It has been suggested that the existence of heterotrophic bacteria in oligotrophic waters is dependent on the association of the cells with particulate material due to the low concentration of organic nutrients and the capability of solid surfaces to concentrate organic nutrients from solution by adsorption (Jannasch and Pritchard, 1972). On the other hand, some workers suggest that, when adsorbed, potential organic nutrients become less available to microorganisms (Smith and Bader, 1961). Sorption reactions have been proposed as a mechanism for preservation of organic material in sediments (Vallentyne, 1962).

There is currently some debate about whether attached

bacteria are more active than planktonic bacteria and about whether attached bacteria are responsible for the majority of the heterotrophic activity in the oceans (Azam and Hodson 1977; Goulder, 1977; Hodson et al. 1981; Harvey and Young, 1980; Kirchmann and Mitchell, 1982). Studies carried out in-situ are difficult to interpret because of the presence of a multitude of organic nutrients and a lack of knowledge about what factors are controlling microbial activity. In addition, the presence of both organic and inorganic particles complicates the picture.

Bacterial attachment to solid surfaces in natural waters is an ubiquitous phenomenon (Jannasch, 1958; Seki, 1970, 1971; Jannasch and Pritchard, 1972; Paerl 1974, 1975, 1980; Seiburth,1975). This attachment leads to a major problem: biofouling. Microbial fouling of surfaces in the marine environment is a major problem in various aspects of ocean technology (O.N.R. Symposium on Biodeterioration in the Marine Environment, 1981; Acker et al. 1972). It is commonly suggested that microfouling is preceded by an organic "conditioning" film on the surface (Baier,1980). However, a true cause and effect relationship between the "conditioning" film and bacterial fouling has not been demonstrated.

If there are advantages to the sessile life of an attached bacterium these may be due to factors other than concentration of nutrients from the solution. Other aspects of the microenvironment at the surface may stimulate

heterotrophic bacteria. These include enrichment of utilizable carbon sources excreted by other microorganisms also on the surface. The use of excreted algal products by bacteria is a well known phenomenon (Chrost, 1978; Chrost and Brzeska, 1978; Chrost and Wazyk, 1978) and may be enhanced by the close proximity of the bacteria and algae on the surface. The proximity of these organisms on surfaces exposed to natural waters has been observed (Marszalek et al. 1979; Sieburth, 1975). Another advantage of attachment may be protection from predation.

An understanding of the mechanisms by which bacterial interaction with surfaces affects heterotrophic activity and the reason(s) why bacteria attach to inorganic surfaces may be helpful in understanding (and possibly controlling) bacterial fouling and corrosion in marine environments. Bacterial interaction with surfaces is also an important factor involved in processes in a variety of environments including soils, sediments, the rhizosphere and the human body.

2. STATEMENT OF THE PROBLEM

Many reports suggest that inorganic surfaces can stimulate bacterial growth and/or respiratory activity in dilute nutrient solutions (Zobell, 1943; Heukelikian and Heller, 1940; Waksman and Carey, 1935). This phenomenon is hereafter referred to as the "surface effect". Unfortunately, the conditions in many of these experiments were poorly defined so that the particular interactions responsible for the observed phenomena could not be precisely identified nor could the experiments be precisely reproduced by other workers. On the basis of these early experimental results a mechanistic hypothesis was proposed. This was that organic nutrients for the bacteria, and the bacteria themselves were concentrated at the surface by adsorption, thus allowing better growth at the interface than in solution. It appears to be accepted by a number of workers that the mechanism of the "surface effect" is enrichment of organic nutrients on surfaces by adsorption (Shilo, 1980; Duursma, 1967; Fletcher,1979). Some workers believe that the breakdown of organic material in aquatic environments is carried out mainly by attached bacteria (Goulder,1977). There is, however, evidence in the literature that is contradictory to these ideas. This is

discussed further in section 3.

There are two facets to the hypothesis that the "surface effect" is caused by concentration of bacteria and organic nutrients at solid/solution interfaces. These are attachment of the bacteria to the surface and the adsorption of the organic nutrient to the surface. One, both, or neither of these interactions may take place in any given system containing solid surfaces, organic nutrients, and bacteria. Whether or not both interactions are necessary for the "surface effect" to occur, or whether either or both necessarily results in the "surface effect" has not been thoroughly examined.

The "surface effect" observed in various reports may be attributed to one or more of the following factors:

1. Enrichment of adsorbed organic nutrients and bacteria at the interface.

2. Enrichment of adsorbed inorganic nutrients at the interface.

3. The solid surfaces themselves contributing nutrients to the bacteria. This may be the case with clays (which may contribute metals to the solution) or organic particles.

4. Qualitative changes in the organic nutrient caused by adsorption that leads to increased availability to degradative enzymes.

5. Adsorption of exoenzymes along with organic

nutrients and bacteria leading to enhanced contact between enzyme and substrate.

6. Other changes in the microenvironment at the interface, such as pH, that can affect bacterial growth.

The purpose of the present study was to test the first mechanism listed above in a controlled experimental system where the other mechanisms were eliminated as much as possible.

The approach used in this project has been to measure heterotrophic activity in the presence of particles in a well defined system, and to quantitate organic nutrient and bacterial adsorption onto the surfaces used. With these measurements it may be determined whether, for the chosen combinations of bacterium, organic nutrient, particle and medium, adsorption of the organic nutrient and/or the bacteria to the surface alters the rate or extent of microbial utilization of the nutrient. The question that is the basis of this project is: does sorption of an organic nutrient and/or the bacteria to a surface result in altered metabolic activity by the bacteria? It is hoped that an examination of this problem in a well defined experimental system will lead to a better understanding of whether bacterial activity is altered at the particle/solution interface due to attachment of the bacteria and/or due to the adsorption of the organic nutrient. In addition, information about whether the availability of the organic

nutrient is altered by adsorption to the surface may be obtained. It is also anticipated that this research will contribute to a better understanding of why bacteria attach to inorganic surfaces and whether the turnover of the particular organic nutrients studied is likely to be mediated mainly by bacteria attached to inorganic surfaces in natural waters.

3. BACKGROUND INFORMATION

3.1 Activity of Surface Associated Bacteria in Natural Water

Bacteria attach to solid surfaces in aquatic environments (Paerl, 1975, 1980; Sieburth, 1975, 1980; Dempsey, 1981; Corpe, 1980) and in laboratory culture (Fletcher, 1979A; Fletcher and Loeb, 1979; Kaneko and Colwell, 1975; Gordon et al., 1981). A succession of periphytic organisms occurs on surfaces exposed to natural waters (Gerchakov et al., 1976; Marsalek et al., 1979). Little is known about the activity and growth of bacteria on surfaces in natural waters or in laboratory culture.

There is currently a variance of opinion concerning whether attached or free bacteria are more important in mineralization and nutrient regeneration processes in aquatic environments. Experiments employing filtration for size fractionation of micro-heterotrophs reach various conclusions on this point. Azam and Hodson (1977) concluded that more than ninety percent of the heterotrophic uptake of glucose in Saanich Inlet, B.C. was due to unattached bacteria. On the other hand, experiments by Goulder (1977) indicate that glucose mineralization was carried out mainly by attached bacteria. Kirchman and

Mitchell (1979,1982) reported that a small number of attached bacteria account for the majority of heterotrophic activity in brackish and fresh water. They also found that a smaller percent of glucose uptake was due to attached bacteria in salt water than in fresh or brackish water. Harvey and Young (1980) found a greater proportion of particle bound bacteria were actively respiring than planktonic cells in a salt marsh environment. Hodson et al. (1981) reported that although attached bacteria were ten times more active than free bacteria, they accounted for only one to two percent of the total heterotrophic activity. The difference in activity could be accounted for by the difference in cell size. Ruben et al. (1981) reported no effect of clay minerals or glass beads on mineralization rates of pesticides by aquatic bacteria. They also found that glass and montmorillonite reduced the mineralization of benzylamine. The quantitative importance of surface associated bacteria to mineralization processes in oceans and estuaries has yet to be established.

3.2 The Effect of Surfaces on Bacterial Activity

There is some evidence that, in nutrient poor growth media, glass surfaces of relatively low surface area to volume ratio (.03 m^2/ml) enhance bacterial growth and respiration (Zobell, 1943; Heukelikian and Heller, 1940; Waksman and Carey, 1935). There are several potential

interactions which may explain these results. The most commonly accepted explanation is that the organic nutrients used by the bacteria are adsorbed from the solution onto the surface and are thereby concentrated. Due to this increased concentration, the nutrients are thought to be more easily assimilated by the bacteria (Zobell, 1943; Jannasch and Pritchard, 1972). Other explanations consistent with the experimental data are that the bacteria are more efficient at scavenging nutrients from solution or in their metabolic processes when they are attached to a surface. This increased entrapment efficiency may be due to secretion of extracellular polymers for attachment which also serve to entrap nutrients (Costerton, 1981; Lange, 1976; Geesey, 1982). Still another explanation, hypothesized by Kriss (1963), is that refractory organic material in seawater is rendered biodegradable by interaction with the surface. It is important to know more about the mechanism of this "surface effect" if one wishes to understand the factors controlling microbial activity at interfaces in natural waters.

The effect of surfaces on bacterial activities is not well understood in any system. Marshall (1971) reviewed the extensive work done on this subject by soil scientists. Studies have indicated that surfaces can stimulate uric acid decomposition (Durand, 1964A), suppress adenine decomposition (Durand, 1964B), stimulate (Macalla, 1939) and suppress (Madhok, 1937) nitrogen fixation, stimulate nitrite oxidation (McLaren and Skujins, 1963), and stimulate sulfate

reduction (Rubentschik et al., 1936). Goldberg and Gainey (1955) found that the activity of nitrifying bacteria was related to the concentration of unadsorbed ammonium and inferred that the adsorbed ammonium was unavailable to the bacteria.

Reports on the effects of glass beads on bacterial glucose utilization show both positive (Heukelikian and Heller, 1940) and indifferent (Zobell, 1943; Stotzky, 1966) effects on bacterial growth and/or respiration. Estermann and McLaren (1959) reported a stimulation of protein decomposition by adsorption of the protein on kaolinite with both free and attached bacteria. On the other hand, Estermann et al. (1959) reported retardation or no effect on protein mineralization due to various adsorbents including kaolinite. Marsham and Marshall (1981) showed that the protein to clay ratio is important in determining the effect of bacteria/protein interactions in the presence of clays. The critical protein to clay ratios for changes in microbial response were not found to correlate with changes in the affinity of the protein for the clay as determined by the adsorption isotherm. A study by Nealson and Ford (1980) indicated that attachment of a marine manganese oxidizing bacterium was essential to its ability to oxidize manganese. Free bacteria showed little or no ability to oxidize manganese while attached bacteria readily utilized this metal. Hattori and Furasaka (1960, 1961) have examined the respiratory activity of Escherischia coli and Azotobacter

agile adsorbed to anion exchange resin and found that attached bacteria are less active. This inhibition was thought to be due to pH differences in the surface microenvironment. This points out the fact that many physical and chemical changes other than adsorption of organic nutrients may occur at the interface and modify microbial activities. Fletcher (1979B) showed that the proportion of actively metabolizing cells on surfaces compared to those in solution was dependent on the type of surface and did not correlate with the tendency of the bacteria to attach to the surface. No simple, generalized hypothesis suffices to explain all the available data that exist on such effects. Even with detailed information regarding specific adsorption reactions between the bacteria, the organic nutrient and the surface, surface areas, surface history, solution chemistry, adsorbent and organisms, it is not presently possible to predict the outcome of such complex interactions.

3.3 Organic Nutrient Adsorption in Relation to the "Surface Effect"

Glucose has been reported to adsorb to quartz and to clays from aqueous solutions (Smith and Bader, 1961; Engel and Holzapfel, 1950; Bondy and Harrington, 1979). Numerous reports exist on the adsorption of amino acids to clays (Smith and Bader, 1961; Stotzky, 1980; Bondy and Harrington,

1979). Unfortunately few experiments were performed in solutions similar to seawater or microbial growth media; most were done in distilled water where competition for surface sites between inorganic and organic chemical species in solution can be neglected. Some workers concerned with biological effects of surfaces have actually made measurements of adsorption of the nutrient of interest to the surface in the growth media used (Zobell, 1943; Stotzky, 1980; Marshman and Marshall, 1981; Goldberg and Gainey, 1955). Others have inferred adsorption of the organic nutrient on the basis of the response of the bacteria (Jannasch and Pritchard, 1972; Heukelikian and Heller, 1940). Zobell (1943) did not observe adsorption of organic nutrients such as glucose, amino acids or peptone to glass. However the work of Heukelikian and Heller (1940) suggests that glucose and peptone do adsorb to glass. This apparent discrepancy is pointed out by Marshall (1976). Due to the many variables involved in such experiments (competition of media constituents, solution pH etc.) it is necessary for adsorption measurements to be made in the experimental media.

The mechanism of adsorption of an organic nutrient to a solid/solution interface must be important in determining its subsequent availability to microorganisms. For example, amino acids and proteins adsorbed by clays are generally found to be less available to soil bacteria (Stotzky, 1980; Gerard and Stotzky, 1973; Estermann et al., 1959). The adsorption of organic matter to particles has

been postulated as a mechanism for preservation of organic matter in soils and sediments (Smith and Bader, 1961; Vallentyne, 1962) and as the first step in the formation of biologically resistant humus (McLaren and Skujins, 1968). However, it appears that when organic substrates adsorb to a surface such as glass where no intercalation would be expected, they are readily available to the organisms (Zobell, 1943; Heukelikian and Heller, 1940).

4. EXPERIMENTAL RATIONALE

4.1 Selection of the Bacterial Strains

Initial experiments were performed using a strain of Vibrio anguillarum selected because it is a marine (or estuarine) bacterium which was readily available. This bacterium is also known to adhere to surfaces (Kaneko and Colwell, 1975).

For the purpose of subsequent experiments a bacterial strain was selected which attached to surfaces exposed to seawater, grew easily in laboratory culture media, and which could utilize a variety of organic substrates. A collection of periphytic marine bacteria isolated from metal and glass surfaces exposed to Biscayne Bay sea water was available (Gerchakov et al., 1976). A bacterial strain was selected from among these isolates on the basis of being representative of a large group of isolates depicted by a computer generated dendogram. The dendogram was based on a large number of biochemical and physiological tests. The characteristics of the selected strain are summarized in Table 1. The organism was identified on the basis of API 20E (Analytical Profile Index, Analytab Products) as Vibrio alginolyticus. The results of these tests are shown in Table

Table 1. CHARACTERISTICS THE Vibrio alginolyticus STRAIN (1)

Gram stain	-
Oxidase	+
Shape	rod
length(microns)	2.0 - 2.4
width(microns)	1.0 - 1.4
motility	+
M - O/F (2)	
Glucose	+/+/-
Galactose	-/-/-
Maltose	+/+/-
Lactose	-/-/-
Xylose	-/-/-
Arabinose	-/-/-
Ribose	+/+/-
Mannose	+/+/-
MacConkey	+
TCBS	Yellow
Pseudocel	-
PIA	+
SS	+
Rimler-Shotts	Green-yellow
Growth Temperature, ^{o}C	
4	-
15	+
25	+
37	+
42	+
Growth NaCl, %	
0	wk
3	+
7	+
9	+
11	+
Novobiocin	Sensitive
O/129 Vibriostat	Resistant
Penicillin	Resistant

(1) + means growth or a positive test result
- means no growth or a negative result
wk means a weak growth

(2) M - O/F 1/2/3: 1-oxidative growth on the sugar
2-fermentative growth on the sugar
3-gas production

Table 2.
RESULTS OF API20E ON THE *Vibrio alginolyticus* STRAIN

TEST	PURPOSE (1)	RESULT
ONPG	beta galactosidase	-
ADH	arginine dihydrolase	-
LDC	lysine decarboxylase	+
ODC	ornithine decarboxylase	+
CIT	citrate	-
H2S	sulfide from thiosulfate	-
URE	urease	-
TDA	tryptophane deaminase	-
IND	indole from tryptophane	+
VP	butanediol fermentation	-
GEL	gelatinase	-
GLU	acid from glucose	+
MAN	" " mannitol	+
INO	" " inositol	-
SOR	" " sorbitol	-
RHA	" " rhamnose	-
SAC	" " sucrose	+
MEL	" " melibiose	-
AMY	" " amygdalin	-
ARA	" " araginose	-
OXI	cytochrome oxidase	+

(1) for further explanation see API20E instructions and references therein

2.

4.2 Selection of Solid Surfaces

Selection of the surfaces used was made at two points in this project. The initial criteria for selection of the surface used were that it be a well defined, highly pure solid that was reported to adsorb low molecular weight, soluble organic compounds that could be utilized by the bacteria as sole carbon and energy sources. In addition, it was deemed desirable for the solid to have little or no reactivity in the experimental medium. In light of these constraints the material selected for initial experimentation was quartz. After initial experiments with the quartz showed that this solid did not adsorb the selected organic nutrients from the selected growth medium, it was apparent that more surface types should be examined. These were selected on the basis of their ability to adsorb the organic nutrients of interest from the experimental medium. The additional material selected was hydroxyapatite.

4.3 Selection of Experimental Medium

The choice of medium was made on the basis of several considerations. The medium had to be suitable for the growth of the bacteria, be well defined especially in terms of organic content, and the organic nutrient had to be the only

limiting factor to bacterial activity. This last criterion was important so that complications due to concentration of inorganic nutrients at the surface could be avoided. The media tested were two defined minimal media and U.V. irradiated Gulfstream water with and without added inorganic nutrients. The medium selected was a defined minimal medium. The experiments which provided the data on the basis of which this selection was made are described in the results section.

4.4 Selection of the Organic Nutrients

The organic nutrients used in these experiments were selected on the basis of being readily utilized by many heterotrophic marine bacteria, of being found in ocean waters, of having been used in experiments where the "surface effect" was reported and of having been reported to adsorb to surfaces. In addition, it was decided that the organic nutrients used should be monomeric so that the complication of involvement of extracellular enzymes would be avoided. The organic compounds selected were glucose and glutamic acid. These compounds fit the criteria listed above and in addition are commom test substances for measuring heterotrophic activity (Wright, 1974; 1978).

Glucose is the dominant monosaccharide in seawater (Mopper et al., 1980). It is found in seawater at concentrations ranging from undetectable to approximately

one micromolar (Vaccaro et al., 1968; Mopper et al., 1980). Free amino acids have similar concentrations as free monosaccharides in seawater (Stumm and Morgan, 1981). However, concentrations of specific amino acids are lower than those reported for glucose (Lee and Bada, 1975). For the purpose of this study, organic substrates were used in the concentration range of 0.1 to 10 micromolar. Some of the earliest experiments performed in this study used glucose at concentrations of 5-16 millimolar.

4.5 Selection of Techniques for Measurement of Bacterial Activity

There are experimental difficulties involved when attempting to make measurements of bacterial activity in suspensions of particulate material where adsorption of organisms and end products may occur. A further complication with such experiments is that anaerobiosis may occur at interfaces. It is desirable to have an experimental technique for monitoring bacterial activity which allows direct measurement of both aerobic and anaerobic metabolism. In addition, the technique should not require radioisotopes since adsorption of the isotope may be a source of error in some procedures. If the requirement for isotopes can be eliminated many types of organic substrate (even undefined, natural isolates) may be used without requiring special synthesis.

4.5.1 Bacterial calorimetry

Microcalorimetry meets the criteria mentioned above. It was therefore selected as a technique for this study.

The use of calorimetry to study heat production by living organisms is not a new development. As long ago as 1790 calorimetry was used by Lavoisier to study the heat output of small animals (Lavoisier,1790). The first study reporting quantitative measurement of bacterial heat production was carried out by Dubrunfaut (1856) who measured heat production from the fermentation of 3.5 tons of sugar. Many early studies were hindered by insufficiently sensitive instrumentation. With the development of modern calorimetric equipment which is commercially available, convenient to use and sensitive enough to measure bacterial metabolism, the number of studies employing this technique has grown. A number of workers have used microcalorimetry to study various aspects of the metabolism of bacteria (Belaich and Belaich, 1976A, 1976B; Belaich et al., 1968; Cooney et al., 1968; Dermoun and Belaich, 1979; Dessers et al., 1970). For a more thorough review of biological microcalorimetry the reader is referred to Beezer (1980). Few studies have been carried out with marine bacteria (McIlvane and Langerman, 1977) and, to my knowledge, no previous calorimetric studies have attempted to use nutrient levels found in marine environments with pure cultures of marine

bacteria. Some experiments at low substrate levels have been performed with terrestrial bacteria (Lovrien et al., 1980).

The profile of heat production versus time, or thermogram, of microorganisms has been shown to be sensitive to conditions including: culture history, aeration, and growth medium used (Few et al., 1976; Beezer et. al., 1976; Perry et. al., 1979).

A linear relationship exists between heat production and moles of substrate oxidized in minimal medium with succinic acid as sole carbon source (Belaich,1979). A similar linear relationship was observed with glucose over a wide concentration range using a marine bacterium (Gordon and Millero, 1980).

Bacterial heat production is composed of several components which may be related by an equation as expressed below:

$$\Delta H_m = \Delta H_c + \Delta H_a + \Delta H_w$$

where ΔH_m is the experimentally measured heat production during bacterial growth, ΔH_c is the heat produced by catabolic reactions, ΔH_a is the heat absorbed by anabolic reactions, and ΔH_w is heat generated due to other biological reactions in the system (motility and transport processes). The ΔH_w is generally neglected (Belaich, 1980). This appears to be justified since, in order to measure heat production from transport processes, concentrations of both bacteria and substrate must be greatly elevated over those encountered in most bacterial

cultures for significant heat production to be measured (Long et al. 1975). During fermentative growth the amount of carbon source used for anabolic processes is so small that the ΔH_a is an insignificant contributing factor to the ΔH_m. Under these conditions, then, $\Delta H_m = \Delta H_c$ and if the endproducts of the fermentation are known this value may be calculated (Forrest, 1969; Belaich, 1980). Under aerobic conditions growth efficiencies are greater than under anaerobic conditions (Stanier et al. 1976) and up to 60% of the carbon source may be assimilated by heterotrophic bacteria (Wiebe and Pomeroy, 1979). As a result ΔH_a is a significant factor and if an independent technique for determination of the fraction of carbon between biomass and endproducts is applied in addition to calorimetric measurement then ΔH_a may be determined experimentally (Dermoun and Belaich, 1979).

4.5.2 Radiorespirometry

Radiorespirometry is a well established technique for measuring the respiratory metabolism of bacteria (Wang, 1971). This technique is sensitive enough to use at micromolar organic nutrient levels and lower. It is limited by the fact that radiolabeled organic nutrients must be available and that it does not give continuous data. Since calorimetry is a novel technique for the application for which it was used in the present study and since the organic nutrients chosen for the present experiments were readily

available with carbon-14 label, radiorespirometry was selected as a technique to give an independent experimental check of the data. In addition, the information derived from respirometry combined with carbon-14 assimilation data gives information on the carbon partitioning between cells and respiratory endproducts that cannot be derived from calorimetric data under aerobic conditions.

5. MATERIALS AND METHODS

5.1 Maintenance of the Bacterial Strain

The culture was maintained on Tryptic Soy Agar (TSA) slants. The stock culture was routinely tested for purity by streaking on TSA plates and was periodically retested on API (see section 4.1) to ascertain that no detectable biochemical changes had occurred. The characteristics of the strain never exhibited any variation as determined by API. The thermogram of the strain maintained in this way did not exhibit any variation over the study period.

5.2 Preparation of Liquid Growth Media

The media used in these experiments were prepared as follows:

5.2.1 Minimal salts medium (M9)

Buffer solution was prepared by dissolving dibasic sodium phosphate (0.05 M), monobasic potassium phosphate (0.02 M), ammonium chloride (0.02 M), and sodium chloride (0.09 M) in distilled water. This solution was sterilized

by autoclaving. After cooling, 10 ml/liter of a 4% w/v solution of magnesium sulfate and 0.2 ml/liter of a 1% w/v calcium chloride solution were added aseptically. These solutions had been sterilized separately by autoclaving. The final concentration of these were: 0.002 M magnesium sulfate and 0.0002 M calcium chloride. The organic nutrient was dissolved in deionized water, filter sterilized (0.1 micron Gelman) and added aseptically to make the desired concentration.

5.2.2 Minimal salts medium (M8)

A second defined minimal medium was made for the purpose of determining the effect of decreased phosphorus and nitrogen concentrations on the heat production by V. alginolyticus. This medium consisted of 0.002 M monobasic sodium carbonate, 0.0005 M dibasic sodium carbonate, 0.0004 M dibasic sodium phosphate, 0.0001 M monobasic potassium phosphate, 0.003 M ammonium chloride and 0.257 M sodium chloride in deionized water. Magnesium sulfate, calcium chloride and the organic nutrient were added as in M9.

5.2.3 Seawater medium (SWM)

Seawater medium was prepared by filtering Gulfstream seawater (0.45 micron Millipore) followed by U.V.

irradiation in a 4 liter reactor with a 450 watt mercury vapor lamp (Ace Glass) for 24 hours. To this solution 5 ml/liter of a nutrient solution consisting of 0.096M dibasic sodium phosphate and 0.057M ammonium chloride was added. The resulting solution was filter sterilized (0.1 micron Gelman). The organic nutrients were prepared as for M9. The sterility of the medium was checked by plating on Marine agar (Difco 2216) and by adding 4 ml to 1 ml of MSWYE broth (see recipe below).

5.2.4 Modified seawater yeast extract (MSWYE)

MSWYE medium was used only to test the sterility of seawater medium. The medium consisted of 0.40 M and 0.028 M of sodium chloride and magnesium sulfate respectively. These were dissolved in deionized water and the pH of the solution was adjusted to 7.4 with 0.1N NaOH. Five g/liter of proteose peptone #2 (Difco) and 5 g/liter of yeast extract (Difco) were added to the solution which was then autoclaved.

5.3 Filter Sterilization

Filter sterilization was achieved with standard vaccuum filtration apparatus sterilized by autoclaving. Gelman Tuffyryn membrane (0.1 micron) filters were generally used. Filtered media were tested for sterility by

spread plating on TSA plates or in the case of seawater medium, plating on Difco 2216 marine agar and adding 4 ml of the filtrate to 1 ml of MSWYE.

5.4 Preparation of Particles

Quartz particles were prepared from single grown quartz crystals obtained from Sawyer Research Products, Inc. The crystals were cut with a rock saw to expose the "Z" region which is the purest region of the crystal (Dr. Kinlock of Sawyer Research Products, personal communication). The cut pieces were broken in a hydraulic press. The pieces were crushed in a standard rock crusher and sieved to obtain uniformly sized fractions. The particles were cleaned in concentrated nitric acid and rinsed in deionized water until there was no change in the pH of the rinse water.

Other particles used were obtained from Bio-Rad and were used as supplied or after the manufacturer's recommended preparation procedure for chromatography. The particles were sterilized by autoclaving.

5.5 Preparation of Pipets and Glassware

Flasks used to grow the cultures in liquid medium were triple baffled nephelo culture flasks (Bellco Glass Co.). These were scrubbed with Iodophore (Klenzade),

rinsed extensively with tap water and distilled water and autoclaved (15 psi 220^{0} F for 15 min) before use.

Pipets were soaked in Iodophore, rinsed with tap water, and then distilled water. They were then autoclaved in metal pipet cans with the tops off for 15-20 minutes.

5.6 Preparation of Solid Media

Solid media used were Difco Tryptic Soy Agar and Difco Marine Agar 2216. These were prepared as per the manufacturer's instructions. The medium was then either placed in 15 ml test tubes (5 ml per tube) and sterilized by autoclaving to make slants or sterilized in the flask to pour into presterilized 100 mm plastic petri plates.

5.7 Preparation of Solutions for the Mixing Calorimeter

Solutions for calorimetry experiments were prepared as follows:

5.7.1 Bacterial suspension

Bacteria were grown in the appropriate liquid medium for 24 hours and harvested by centrifugation 1200 X g for 20-30 minutes. The cells were washed twice in the appropriate liquid medium without organic nutrient and resuspended to the desired final absorbance measured in a

Bausch and Lomb spectronic 20 at 520 nM.

5.7.2 Organic nutrients

Organic nutrients were prepared in millimolar concentration ranges in distilled water and then diluted volumetrically 1:1000 in medium generally by using a 100ul Oxford pipet and a 100 ml volumetric flask. This procedure yielded organic nutrients at micromolar concentrations which were then filter sterilized.

5.7.3 Medium pre-exposed to particles

For calorimetry experiments in which hydroxyapatite particles were used, medium was prepared by pre-exposure to the particles so that any removal of inorganic nutrients by adsorption or other changes in the medium would be the same in experiments with and without particles. Medium was added to particles at the same weight to volume ratio as used in calorimetry (section 5.8.2) and adsorption (sections 5.9 and 5.12) experiments. The mixture was shaken and allowed to equilibrate for 2-3 hours. This time was shown to be more than enough for the adsorption of organic nutrients to reach equilibrium (section 6.2) and it was assumed that the inorganic nutrients would adsorb in equivalent times. The particles were allowed to settle and the supernatant was removed with a pasteur pipet. The supernatant was filter

sterilized and transferred aseptically into a sterile bottle.

5.8 Calorimetry Procedures

5.8.1 Tronac calorimeter

A schematic diagram of the Tronac calorimeter is shown in Figure 1. The isothermal reaction vessel is contained in a twenty gallon temperature regulated water bath during experiments. The water bath was maintained at a temperature of 25 $\pm$ 0.01 ^{o}C. The temperature of the contents of the reaction vessel is maintained at a constant temperature ($\pm$ .00005^{o}C) by constant cooling from a Peltier cooler and the addition of heat as necessary with an electrical heater. The input from the heater is inversely proportional to the heat production of the reaction in the calorimeter. The contents of the reaction vessel were constantly stirred. For the purpose of bacterial calorimetry an aeration system was built to supply hydrated, temperature equilibrated gasses to the reaction vessel.

In preparation for an experiment the reaction vessel was treated with Iodophore and rinsed extensively with distilled water. The stirrer passage was rinsed with water and dried with acetone. The reaction vessel was filled with 70% (by volume in water) ethanol and assembled so that the vessel, thermistors and stirrer were immersed. The system was allowed to soak in the ethanol solution for 20 minutes

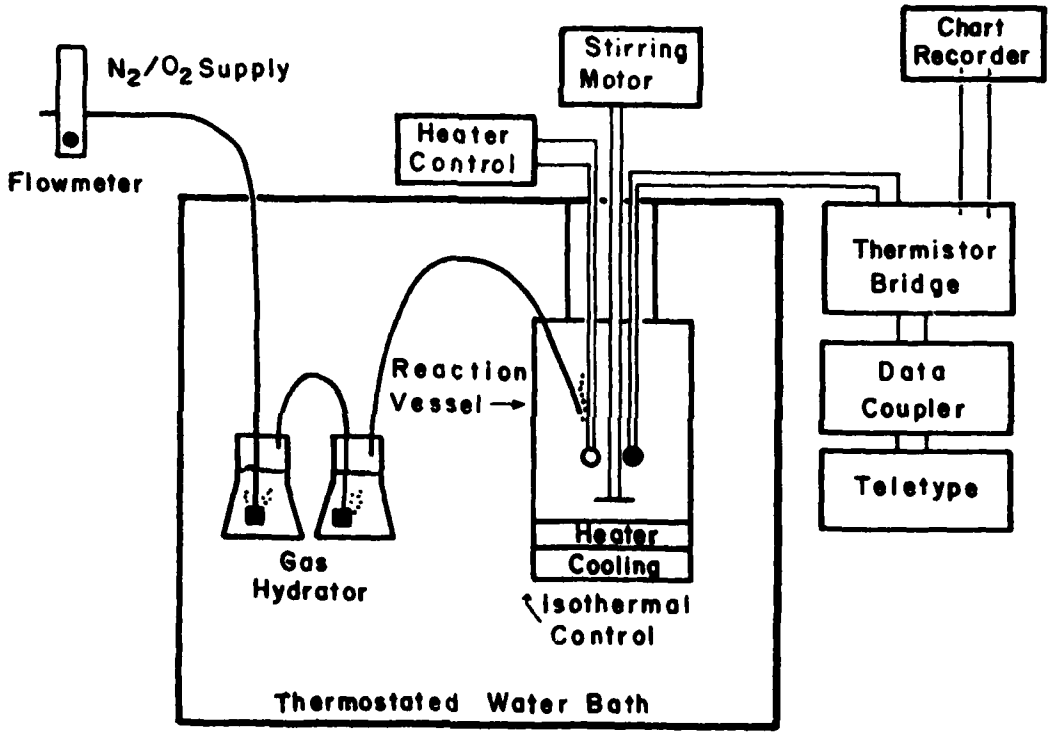


Figure 1. A schematic diagram of the Tronac isothermal titration calorimeter.

with the stirrer on. The reaction vessel was then rinsed 5 times with sterile distilled water and assembled, filled with sterile distilled water. Subsequently, the water was poured out and the vessel was assembled and dried with a stream of sterile air. The bacterial culture was grown in liquid culture to an optical density of 0.1 at 520 nm (Bausch and Lomb Spectronic 20) on a rotary shaker and then placed into the reaction vessel. The data were collected on a strip chart recorder (Hewlett-Packard model 7101B) and when desired on paper punch tape. Aeration was achieved by passing a water saturated, temperature equilibrated air or oxygen/nitrogen mixture through the solution in the reaction vessel with two Teflon tubes. The flow rate was controlled by two SS-2MGD Nupro metering valves. The rate was calibrated with a rotameter (model E29, Air Products).

5.8.2 LKB mixing calorimeter

A schematic diagram of the mixing calorimetry system is shown in Figure 2. This calorimetry system detects heat flow from the reaction vessel, through thermopiles -which convert temperature changes to voltages- and into a metal heat sink. The calorimeter unit is contained in a constant ($\pm$ .01°C) temperature air bath. There are two reaction cells each divided into two compartments. The contents of the separated compartments are mixed by rotation of the calorimeter unit (Figure 2). The voltage outputs from the

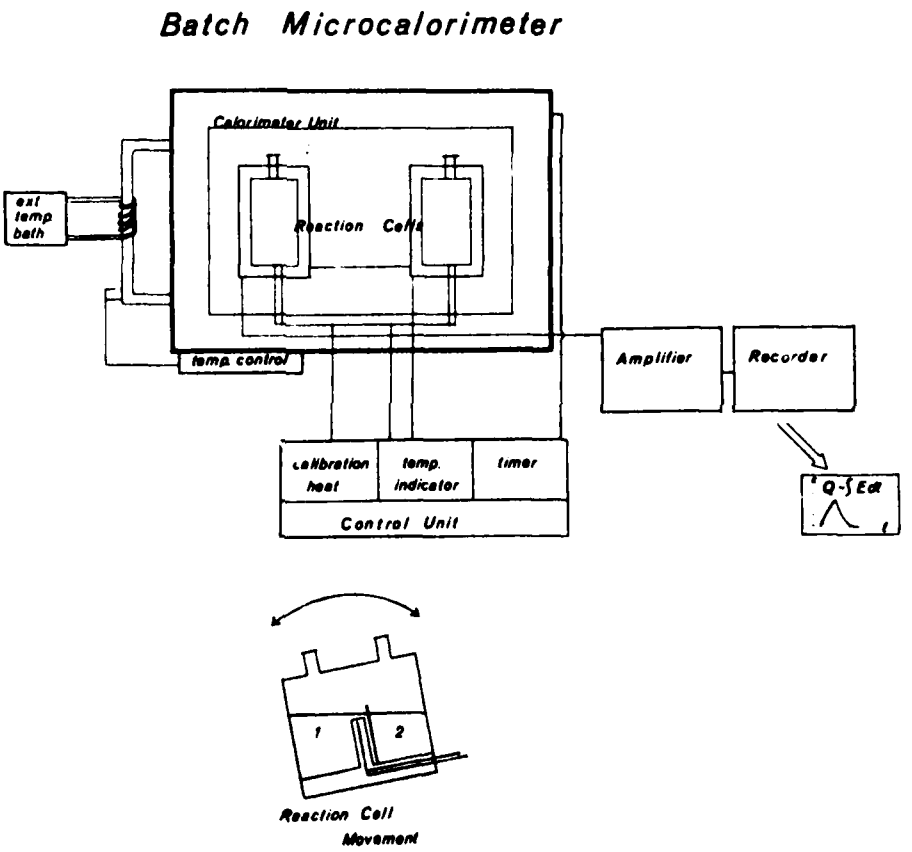


Figure 2. A schematic diagram of the LKB mixins calorimeter.

thermopiles of the two cells are connected in opposition so that the measured signal is the difference between the signals from the two cells. This configuration is referred to as the "twin principle" of this system and allows simultaneous control experiments to be performed as well as helping to eliminate baseline variation due to temperature fluctuations in the constant temperature air bath.

Bacterial cell suspension and solutions prepared as described above were placed in the appropriate chamber of the calorimeter with a sterile 5cc syringe. The precision of the procedure for addition of the solutions was checked by weight of water and found to be reproducible to within $\pm$ 0.01 ml or 0.5%. A stable baseline was achieved in one to two hours and then the reactants were mixed. The data were collected on a Hewlett Packard model 7101B strip chart recorder or on a Heath - Schlumberger model EU-205-11 recorder or processed by the automated data system described in Appendix A. After an experiment the reaction cells were flushed with water to remove particles and solutions, then rinsed with 0.5 N NaOH, 0.1 N HCl, distilled water, soaked 20 minutes in 70% ethanol, rinsed in acetone and dried with a stream of air or nitrogen.

5.9 Organic Nutrient Adsorption

Solutions of radioactive glucose and glutamic acid were procured from New England Nuclear. The specific activity of the glucose was 14 milliCuries/millimole and the glutamic

acid was 292 milliCuries/millimole. These solutions were supplied in ethanol. The ethanol was removed by evaporation (50°C) and the isotope was dissolved in sterile media before use. Solutions of carrier were made and the radiolabel was spiked into the solution to give 5,000-10,000 counts per minute and to bring the final organic nutrient concentration to the desired value. Particles were prepared as for calorimetry experiments. Adsorption experiments were carried out by placing twenty milliliters of medium containing radioactive organic in a sterile, acid washed (dichromic acid) 125 ml erlenmeyer flask with a ground glass stopper. The solution was sampled (100 microliters) and the sample placed in 5cc of scintillation fluid (Aquascint,ICN) in a vial. Two replicates of each sample were taken. The particles were then placed into the solution and the mixture shaken on a rotary shaker with sufficient force to keep the particles in suspension. When a sample was to be taken, one milliliter of the suspension was withdrawn with an oxford pipet with a sterile tip. The sample was placed in a conical 15 ml centrifuge tube and centrifuged in a clinical centrifuge. The supernatant (100 microliters) was sampled for counting. Counting was done in a Packard Tri-Carb liquid scintillation counter (model 3375). The raw counts were corrected for background counts by subtraction.

5.10 Scanning Electron Microscopy

Samples for scanning electron microscopy (S.E.M.) were prepared by exposure of the solid of interest to a growing culture of the bacterium of interest. After incubation for the designated time the particles (or glass coverslips) were removed from the culture by filtration onto Teflon filters (millipore LC, 10 micron pore diameter) or, in the case of coverslips, with tweezers. The samples were then rinsed with M9 without organic nutrient to remove non-attached bacteria and fixed in 4% glutaraldehyde in M9 for 15 minutes. The samples were rinsed in M9 buffer and then treated with the following solutions for 10 minutes each:

25% distilled water (D.W.) 75% M9 buffer

50% D.W. 50% M9 buffer

75% D.W. 25% M9 buffer

100% D.W.

50% D.W. 50% Acetone

100% Acetone

50% Acetone 50% Xylene

100% Xylene

The samples were then air dried, placed on sample holders, and coated with gold-palladium in a vacuum evaporator. The samples were examined with a scanning electron microscope (ISI, model DS 130) using an accelerating voltage of 30 kv.

5.11 Surface Area Measurement

Surface areas were measured with the apparatus of de Kanal and Morse (1979). The technique is essentially that of Brunauer, Emmett, and Teller (1938). Surface area is determined from the adsorption isotherm of krypton to the surface. The method employs a three point determination of ratios of the equilibrium gas pressure at room temperature and at the temperature of liquid nitrogen. The surface area of the sample may be calculated from the linear regression analysis of data points derived from these ratios. These calculations are described in detail by de Kanal and Morse (1979).

5.12 Bacterial Attachment

Bacterial attachment to hydroxyapatite was assayed by a modified version of the technique of Gibbons, Moreno and Spinell (1976). The assay involves incubating radiolabelled bacteria with the solid of interest and measuring the decrease in radioactivity in the solution as the bacteria become adsorbed to the surface. Bacteria were grown to an optical density of 0.2 A (log phase) in M9 and then harvested by centrifugation (12000 x g for 20 minutes). The cells were washed once with the medium and then resuspended in medium containing 5 micromoles/liter of uniformly labelled C-14 glucose (14 milliCuries per millimole). The bacteria were incubated for 1 hour. After this time, plate

counts were made and the bacterial suspension was diluted with medium to make suspensions with various concentrations of bacteria. One milliliter aliquots of the suspensions were added to test tubes (15 ml, 16 mm OD) containing 0.125 gram of hydroxyapatite powder. The tubes were incubated at room temperature on an angle on a rotary shaker for 18 hours. After the incubation period the tubes were removed from the shaker and the particles were allowed to settle. Radioactivity retained on a 0.45 micron pore (Millipore HA) was assayed from 0.1 ml of the supernatant and 0.1 ml of the total slurry. These values were compared to values from control tubes without particles to determine the decrease in counts in the solution due to adsorption of the bacteria. Plate counts of the supernatant from the tubes containing particles were made occasionally to check for agreement with bacterial numbers predicted from the radioassay.

5.13 Radiorespirometry

Radiorespirometric measurements were made with glucose and glutamic acid as sole carbon sources in the presence and absence of hydroxyapatite particles. The experiments were carried out by a technique similar to that used by Cooksey (1972, 1974). Ten milliliter respirometry flasks with center wells (Kontes) were used for this experiment. Two different procedures were used for these experiments depending on whether metabolism by attached bacteria or bacterial metabolism of organic nutrients pre-exposed to particles

was to be examined.

The metabolism of organic nutrients pre-exposed (but not necessarily adsorbed) to hydroxyapatite was measured by the following procedure. Medium containing 6.7 micromolar organic nutrient was exposed to the particles (0.125 g/ml) for one hour on a rotary shaker. After this time 0.33 ml of bacterial suspension (section 5.7.1) was added. The final concentration of organic carbon source was 5 micromolar and the final concentration of bacteria was $6*10^7$/ml. Sodium hydroxide (0.1 ml of 10% w/v) was added to the center well and the serum caps sealed. Flasks with and without particles were prepared in duplicate. The experiment was carried on for one hour and then 0.3 ml of 85% phosphoric acid was added and the flasks were replaced on the shaker for 1/2 hour. This concentration of acid dissolves all hydroxyapatite so that it cannot retain any carbon dioxide. The contents of the center well were removed and placed in a 10 ml scintillation vial containing 5 ml Aquascint (ICN). The well was rinsed 3 times with 0.1 ml of distilled water and the rinse water placed into the vial. The culture solution (0.1 ml) was also placed in a scintillation vial. The vials were assayed for radioactivity as described previously.

Metabolism by attached bacteria was measured by the following procedure. Bacteria attached to hydroxyapatite were prepared by the procedure previously described for the attachment assay (section 5.12) except that the bacteria

were not radiolabeled. Bacteria in flasks without particles were also prepared as a control. A 1:500 dilution of the washed culture was used for the experiment; this yielded a suspension of $6.2 \times 10^5 \pm 0.8 \times 10^5$ bacteria per milliliter as determined by plate count. At this concentration 85 to 87% of the bacteria become attached to the surface (see section 6.3). After exposure to the particles, uniformly labeled carbon-14 glucose or glutamic acid was added at the desired final concentration. The experiment was carried on for 24 hours and the contents of the NaOH trap and the solution were harvested and assayed for radioactivity as previously described.

5.14 Carbon Assimilation

Incorporation of carbon from glucose or glutamic acid into cell material was measured by the following procedure. Attached bacteria and control flasks were prepared as previously described (5.13). After addition of the radioactive substrate, the cultures were incubated for 24 hours and then fixed with 4% glutaraldehyde (final concentration) for 15 minutes. An aliquot of the culture was added to 5 ml of Aquascint in a 10 ml scintillation flask; another aliquot was filtered (0.1 ml culture through a 0.45 micron millipore HA filter) and the filtered material was rinsed with three 0.1 milliliter aliquots of medium. The filter was placed into scintillation fluid and dissolved by

shaking. Radioactivity was assayed as previously described.

5.15 Gas Chromatography

The procedure used for analysis of low molecular weight carboxylic acids in the media was that of Sanalitro and Muirhead (1975). Analysis of butyl-esters of the acids was performed on a Tracor model 560 gas chromatograph with a flame ionization detector. A Hewlett-Packard 3385A integrator/recorder recorded the chromatograms, integrated the peaks and recorded the retention times. The retention times and peak areas were compared to those of standards previously injected. The standards solution contained formic, acetic, propionic, isobutyric, lactic, isovaleric, valeric, caproic, heptanoic, oxalic, malonic and succinic acids (40 mM).

6. RESULTS

6.1 Determination of Growth Limiting Factors in the Media

The factors controlling the metabolism of bacteria in the various growth media were determined by calorimetry. These preliminary experiments were performed since it was necessary that the organic nutrient be the only factor limiting to bacterial metabolism for the purpose of testing the hypothesis that bacterial activity at interfaces is enhanced due to adsorption of organic nutrients and bacteria.

6.1.1 Millimolar levels of organic nutrient

Initial studies using the Tronac calorimetry system showed that aeration rates were important factors controlling the shape of the thermogram produced by a bacterial culture. The effect of different aeration and oxygenation rates on the thermogram of Vibrio anguillarum in M9 with 16 mM glucose was examined. Both the integrated heat production and the rate of heat production are greater during aerated growth than during non-aerated growth (Figure 3). In addition, the thermogram produced during aerated

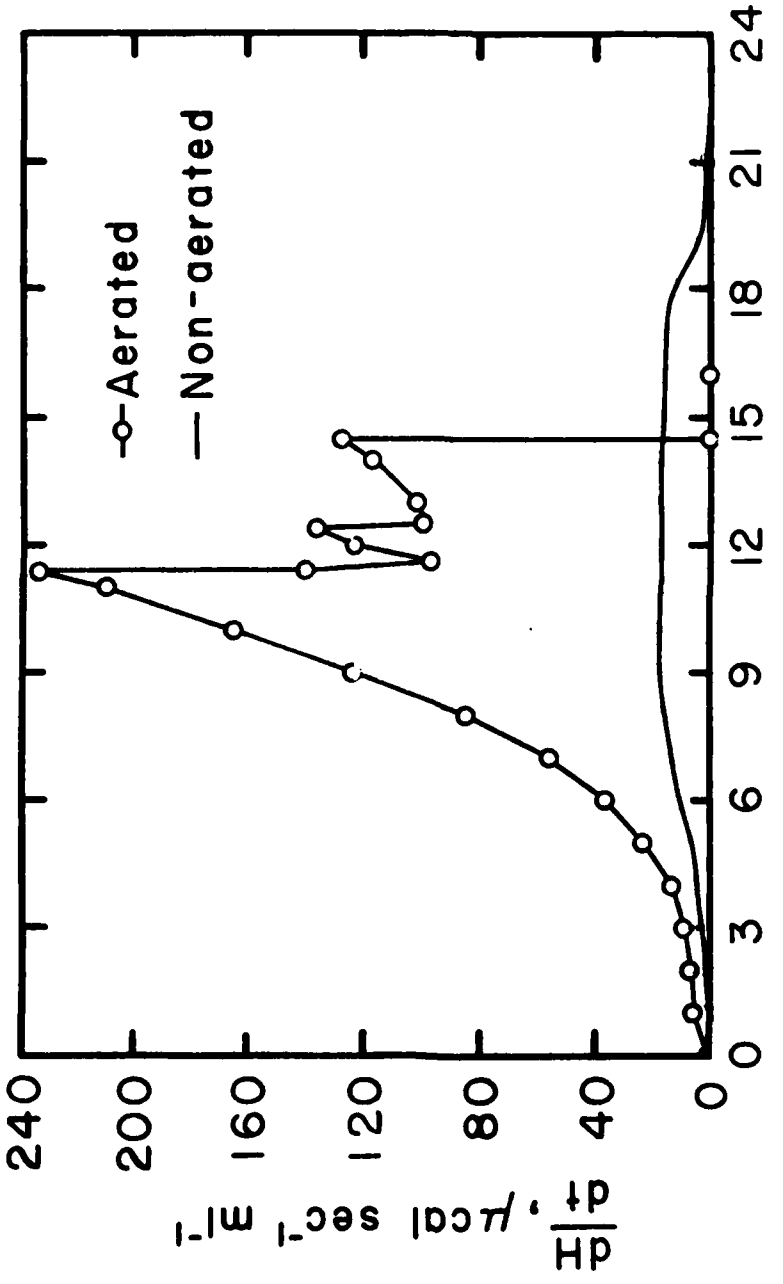


Figure 3. The effect of aeration on the thermogram produced by Vibrio anguillarum in the Tronac calorimeter. Glucose (16 mM) was used as the sole carbon and energy source in M9 medium. Aeration was achieved using air at a flow rate of 5 cc/min.

growth has three distinct peaks. The fact that heat production is diminished by lack of aeration would be expected since these facultatively anaerobic bacteria can ferment their substrates when oxygen is limited. Fermentation leads to a diminished heat production per mole of substrate. The heat generated during fermentative growth is found to be in the range of -23 to -60 kcal per mole of mono- or disaccharide (Belaich, 1980) whereas aerobic growth yields approximately 50% of the heat of combustion of the substrate (Dermoun and Belaich, 1979; Gordon and Millero, 1980). Increasing the oxygen concentration in the inflowing gas supply to the calorimeter was found to affect the shape of the thermogram (Figure 4). The secondary peaks disappear at high oxygen concentrations (100% O_2). This suggests that the secondary peaks may be due to incomplete oxygenation at the lower oxygen concentrations and shows that the oxygen concentration is an important factor controlling the metabolism of the bacteria in this system.

There is a linear correlation between bacterial numbers and the rate of heat production during exponential growth (Figure 5). This has been previously shown by other workers (Forrest, 1969). The slight curvature that can be seen in the data (Figure 5) may be explained by limitation of oxygen during the latter stages of growth leading to a decrease in heat production as the culture begins to become anaerobic.

Secondary peaks in the aerated thermogram were shown to

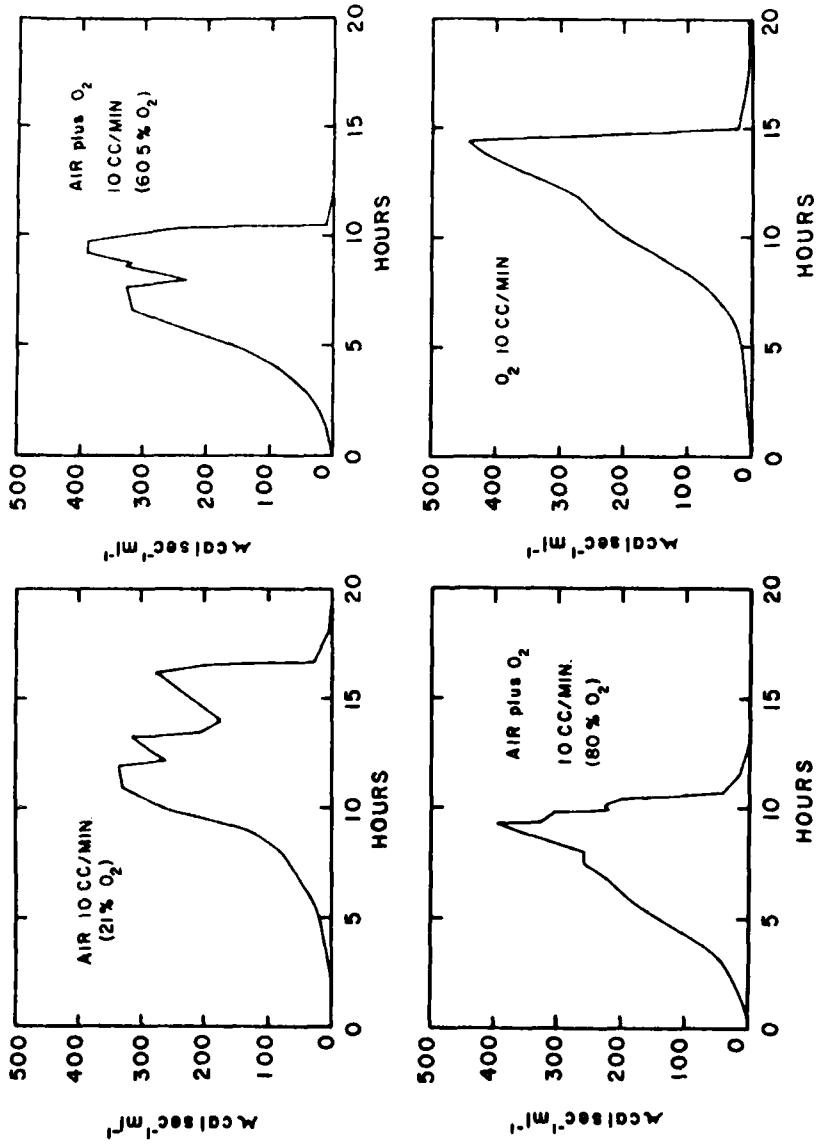


Figure 4. The effect of various oxygen concentrations in the inflowing gas stream on the thermogram of Vibrio anguillarum. The glucose concentration was 16 mM in M9. The % O_2 in the O_2/N_2 mixture is indicated in parentheses.

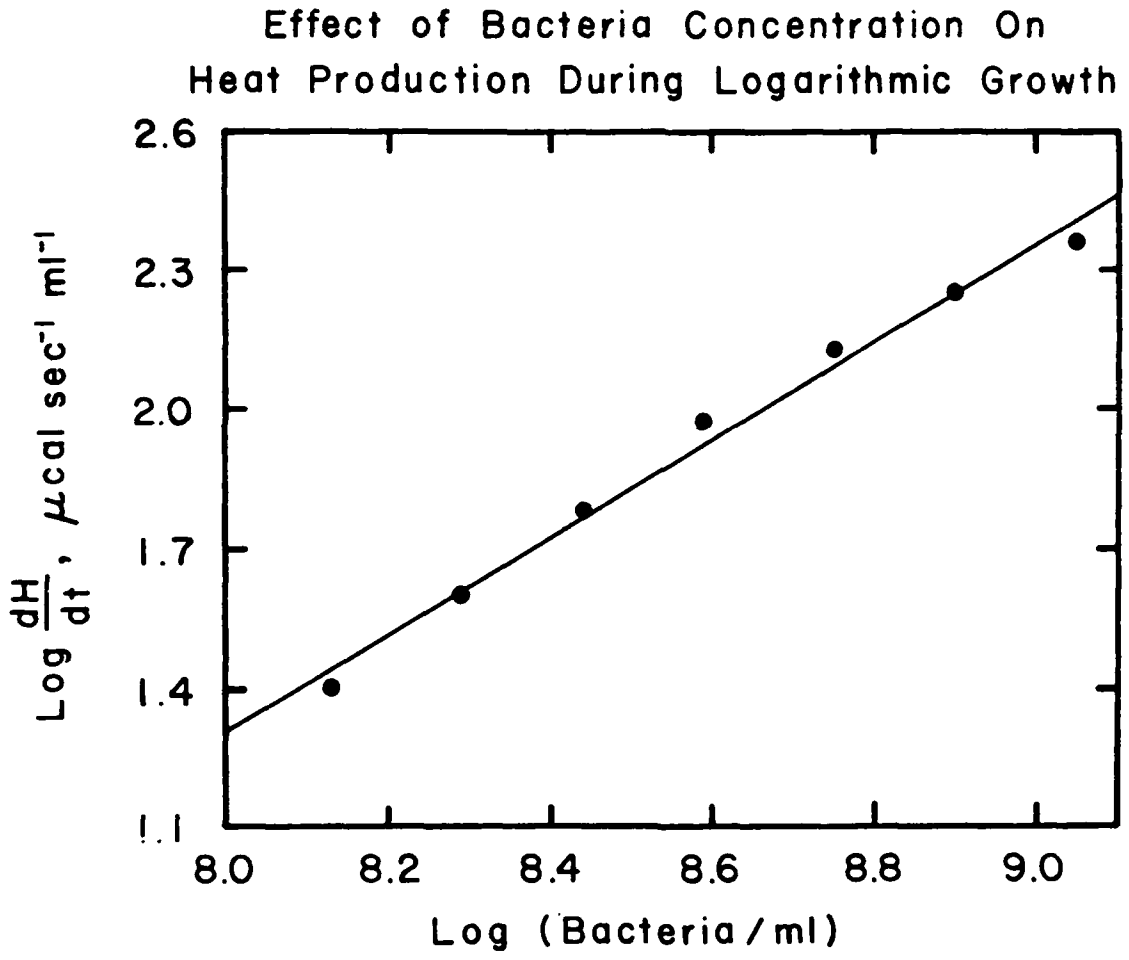


Figure 5. The correlation between bacterial concentration (measured by plate counts) and the rate of heat production during exponential growth. Vibrio anguillarum was grown in the Tronac calorimeter for this experiment.

be caused by accumulation and subsequent degradation of acetic and lactic acids (Figure 6). This observation again suggests that oxygen limitation may be occurring in the calorimeter. The fermentation products were analyzed by gas chromatography.

The concentration of glucose was found to control the area but not the shape of thermograms (Figures 7 and 8). Figure 7 is a plot of the areas of the thermograms shown in Figure 8. The area (integrated heat) of the thermogram is directly related to the amount of glucose added to the calorimeter (Figure 7). The slope of the linear regression line gives a value of -304.3 kcal/mole for the heat produced from metabolism of glucose under these conditions or 45% of the heat of combustion of the substrate. The general shape of the thermogram is maintained at the lowered glucose concentrations (Figure 8). This suggests that the growth rate, as indicated by the rate of heat production during exponential growth, is not diminished by lowering the glucose level. Glucose is therefore limiting the total heat production indicating that it is growth limiting. Since oxygen was also found to be controlling the metabolism of the bacteria, it was apparent that the oxygen level as well as the glucose concentration was affecting bacterial metabolism in this system. This experimental set-up was therefore not ideal for measuring the effect of particles on the metabolism of organic nutrients by bacteria since the organic nutrient was not the only factor controlling

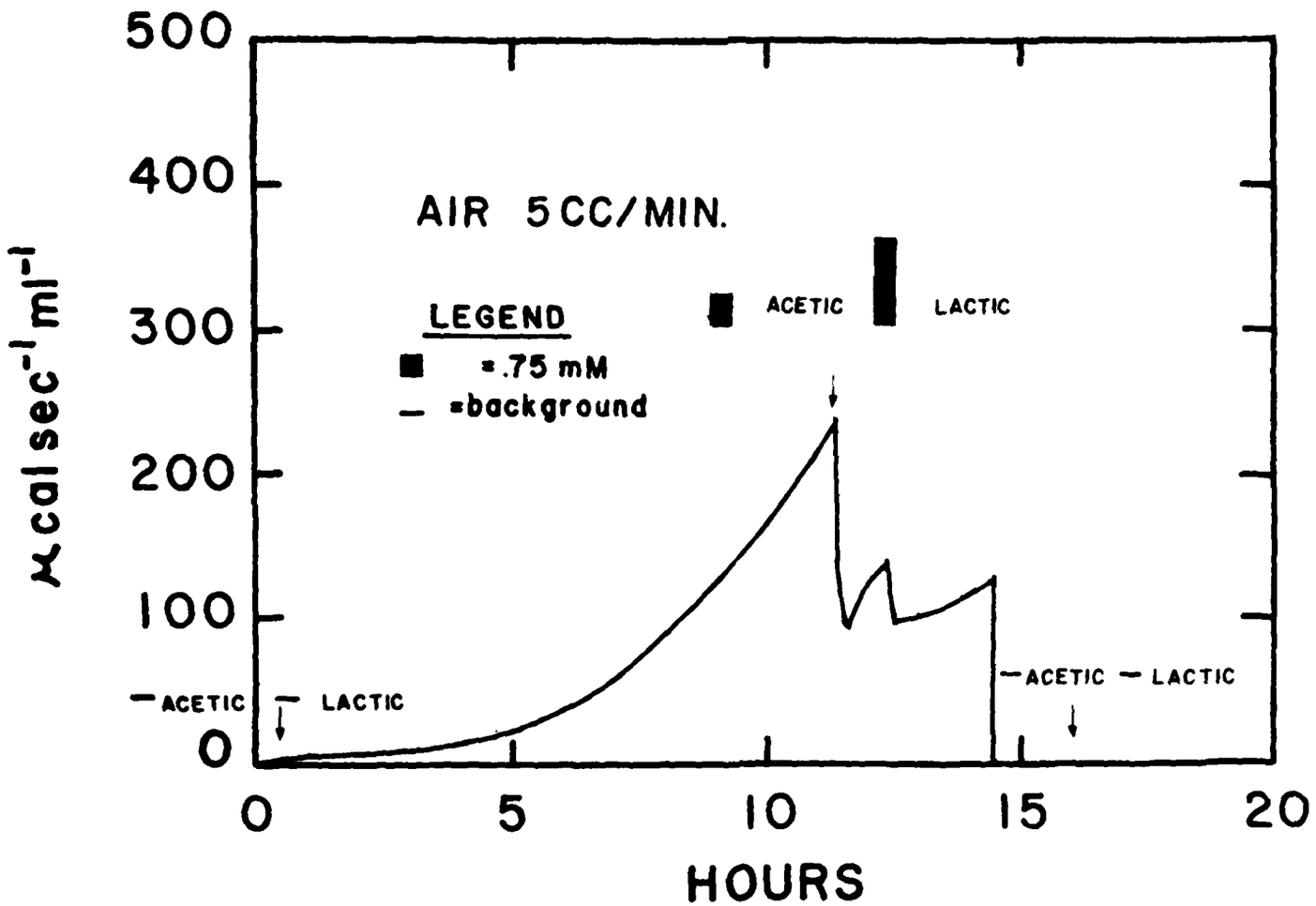


Figure 6. Accumulation and degradation of fermentation products during the growth of Vibrio anguillarum in the Tronac calorimeter. The medium used in this experiment was M9 with 16 mM glucose.

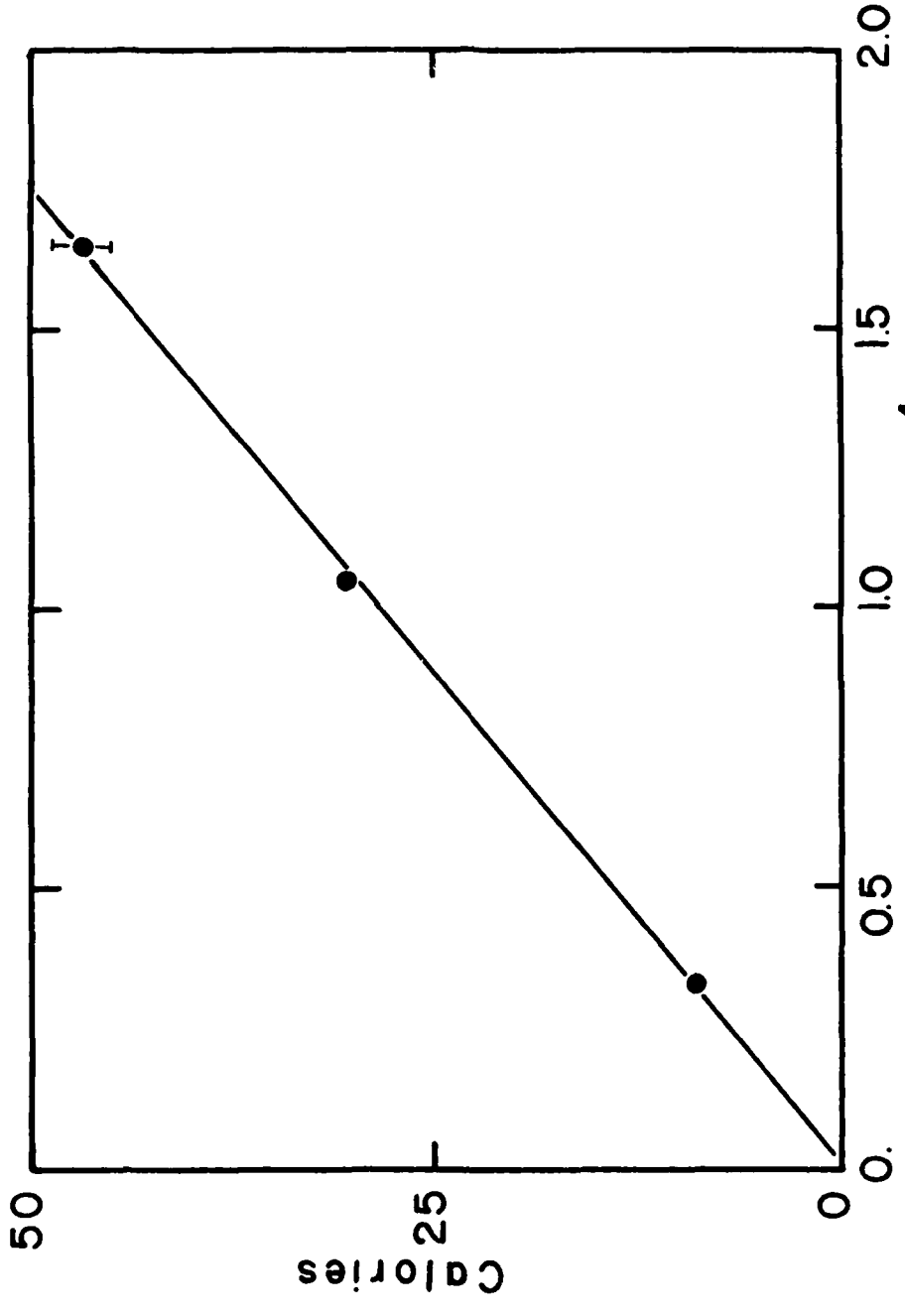


Figure 7. The effect of glucose concentration on the heat produced from glucose by Vibrio alginolyticus in the Tronac calorimeter.

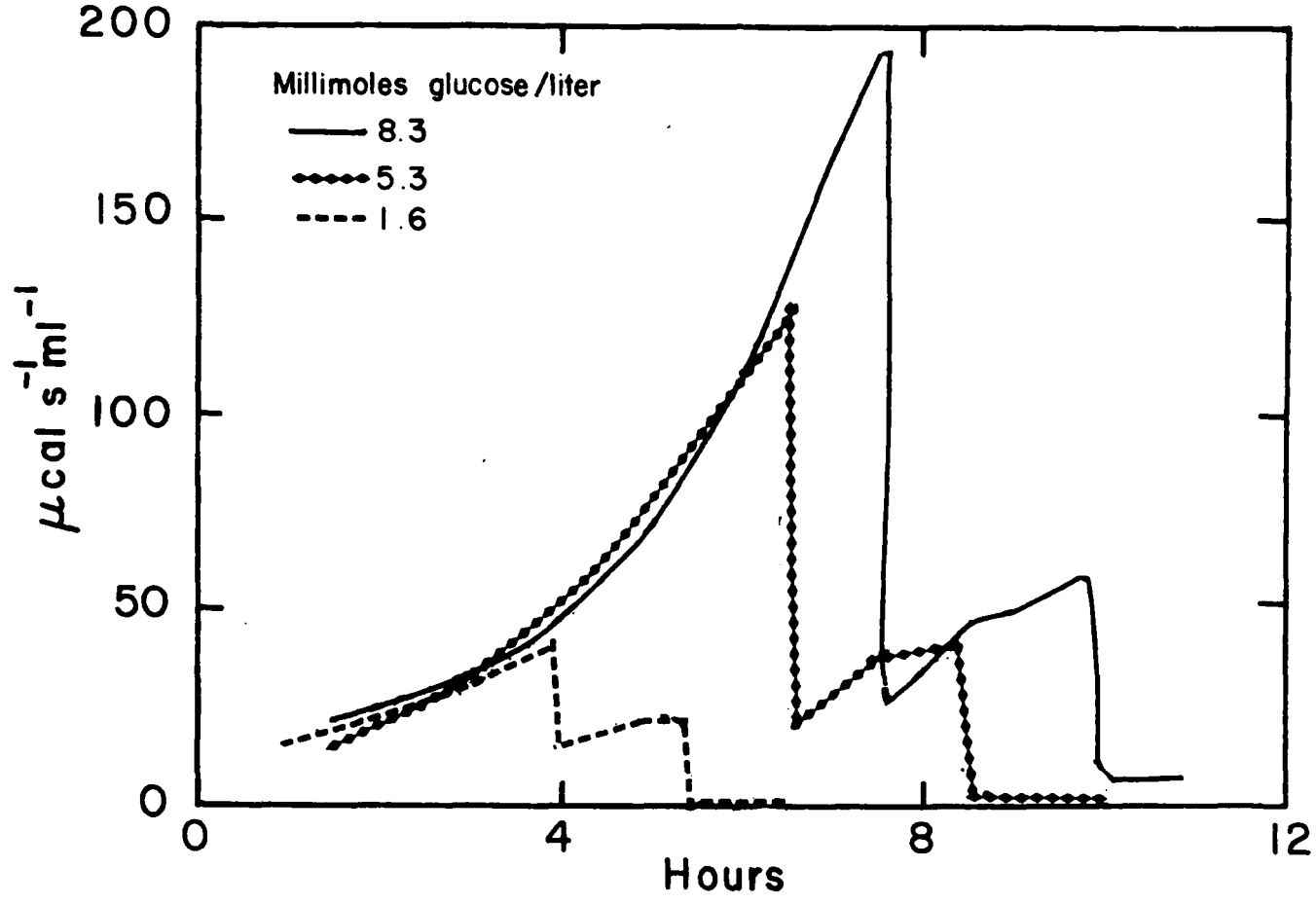


Figure 8. Thermograms produced by Vibrio alginolyticus in the Tronac calorimeter with various glucose concentrations in M9.

metabolic activity. In addition, the high level of organic nutrient was not representative of natural waters. For further information and discussion about the use of calorimetry to study the growth of marine bacteria the reader is referred to the appendices.

6.1.2 Micromolar levels of organic nutrient

Because of the limitations of the experimental system employing the Tronac calorimeter the applicability of another available calorimetry system with higher sensitivity (an LKB batch calorimeter) was examined. It was hoped that this system would have a high enough sensitivity to allow measurements to be made at concentrations of organic nutrient which were at or near natural levels so that oxygen concentrations would not be limiting and the experiments would be more representative of natural conditions.

The experimental procedure employing the LKB batch calorimeter was found to be useful for glucose concentrations as low as 3×10^{-7} M when concentrations of bacteria were $5\text{-}10 \times 10^{7}$ bacteria /ml in a 6 ml sample (Gordon et al., 1981). Due to the low glucose concentration in these experiments the thermograms generated in this system had a much shorter time span than those in the Tronac system (Figure 9). Integrated heats plotted as a function of time were calculated and plotted by a computer (Figure 9 and appendix A). The heats in this system were also found to be a linear function of glucose concentration over the

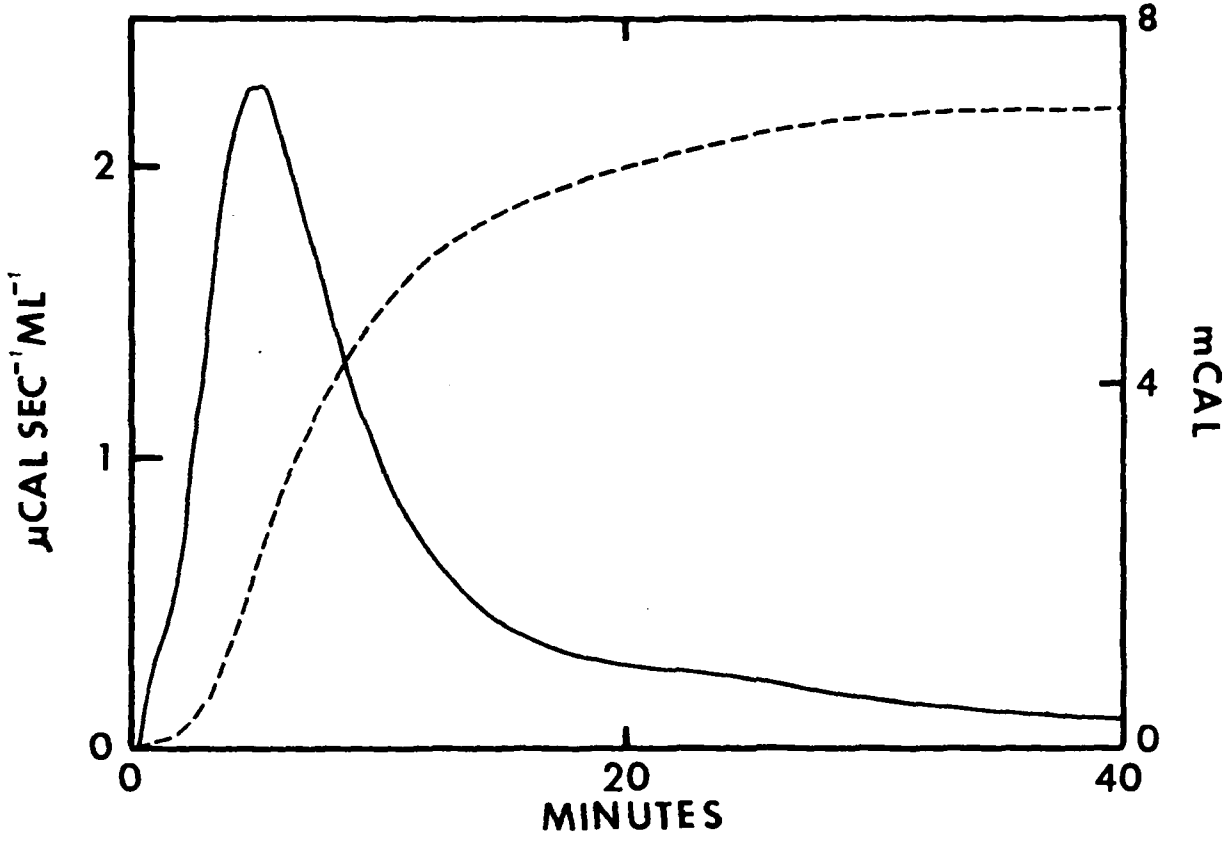


Figure 9. A thermogram (solid line) and integration of the thermogram (dashed line) of Vibrio alginolyticus. This experiment was performed in the LKB mixing calorimeter using M9 with 5×10^{-6} M glucose. Bacteria were starved overnight and resuspended in medium at a concentration of 6×10^{7} bacteria/ml. Heat was generated when bacteria were mixed with a glucose solution.

concentration range studied (Figure 10).

The LKB calorimetry assay was used for examining the effect of different minimal growth media on the heat production of marine bacteria in order to select an appropriate medium for subsequent experiments. It was originally intended that the experiments with adsorbed organic nutrients and bacteria would be carried out in seawater medium. However, it was found by investigation of heat production in several media that inorganic nutrient concentrations that can be achieved in natural seawater medium may affect the metabolic activity of the bacteria at the organic nutrient concentrations used. Differences in total phosphate and nitrogen concentrations in the media tested are shown in Table 3. Their detailed compositions are given in section 5.2. As can be seen from the heat production in the various media (Figure 11) the kinetics of heat production are different in seawater (SW), seawater medium (SWM), M8, and M9. Heat production in SWM and M8 is slower in reaching completion than in M9 but the total heat produced is the same in all three media. M8 and SWM have the same nitrogen and phosphorus concentration, which is one hundred fold lower than M9. This could explain the slower kinetics of heat production observed in these media. Heat production in seawater with no added inorganic nutrients is even lower than seawater medium or M8. The total heat production as well as the rate is lessened in SW. Again this can be explained in terms of lower inorganic nutrient

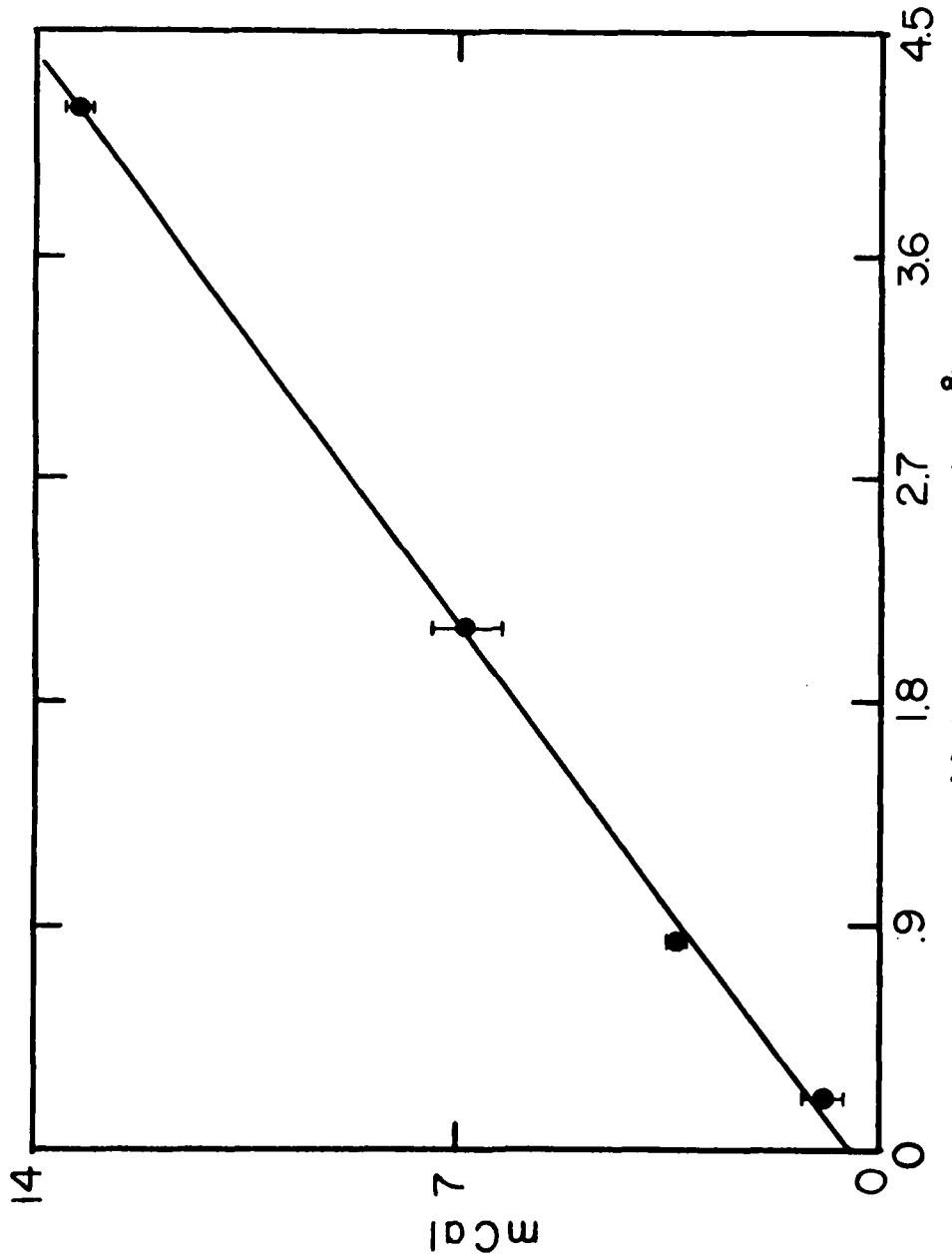


Figure 10. Total heat produced by Vibrio alginolyticus as a function of glucose concentration in the LKB mixing calorimeter.

Table 3. PHOSPHATE AND NITROGEN CONCENTRATIONS IN THE MEDIA TESTED.

Medium (1)	Total Phosphate	Total Nitrogen
M9	0.07 M	0.02 M
M8	0.0005 M	0.003 M
SWM	0.0005 M	0.003 M
SW	<0.000001 M (2)	<0.000001 M

(1) see section 5.2 for detailed composition
(2) from J. Morse and J. Zullig (unpublished data)

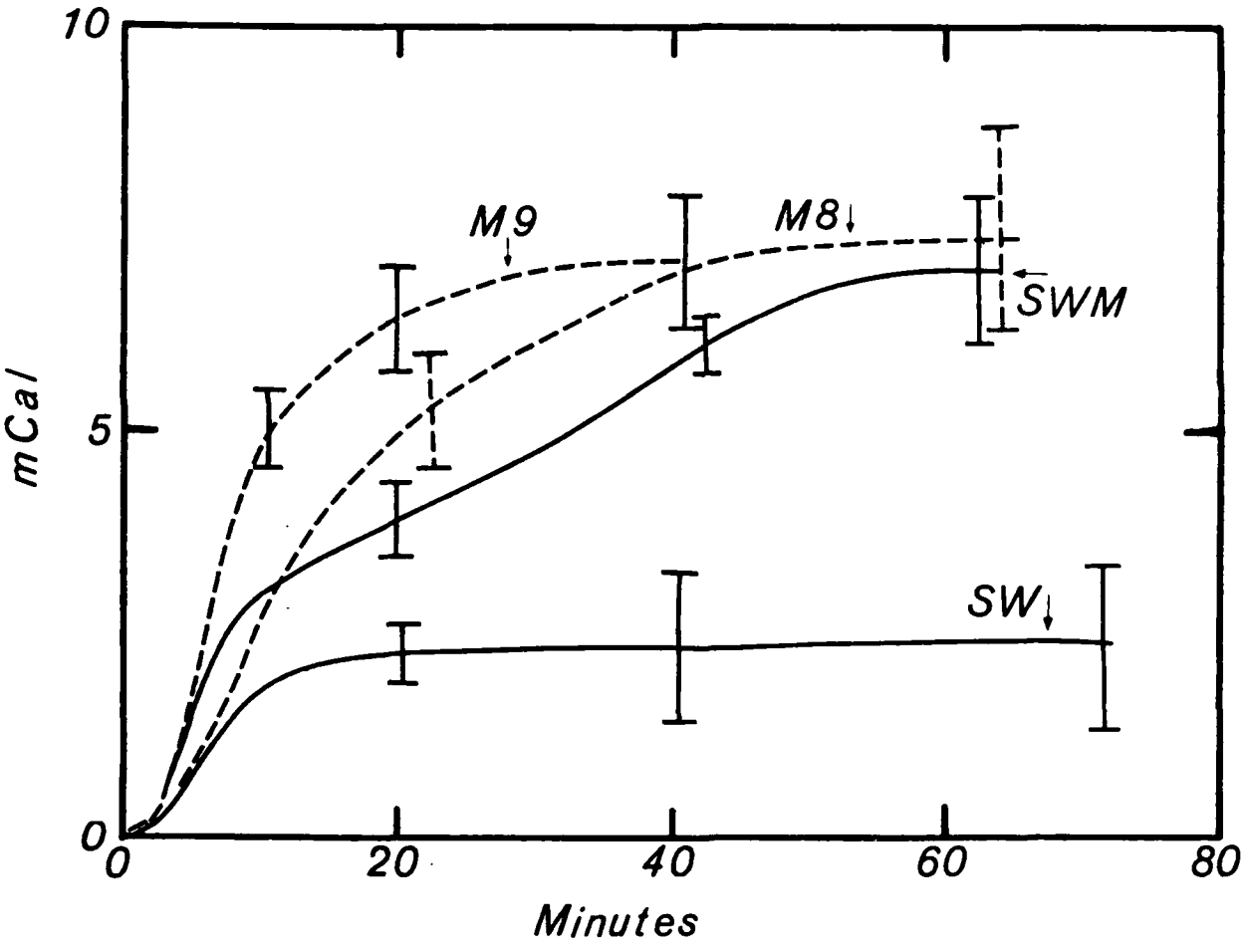


Figure 11. Comparison of heat production from 5 x 10^{-6} M glucose by Vibrio alginolyticus (6 x 10^{7} bacteria/ml) in various defined media.

levels (Table 3). M9 medium was used in all subsequent experiments to avoid complications by nitrogen or phosphorus limitation.

6.2 Adsorption of Glucose and Glutamic Acid onto Particles

Adsorption experiments indicated that the tendency of glucose and glutamic acid to become associated with the inorganic particles tested in either M9 or seawater was not great. Experiments performed with organic ion exchange resins yielded higher adsorption; however, the values obtained in either M9 or seawater in this study were lower than values reported for similar studies in distilled water solutions (Smith and Bader, 1961; Kresak et al., 1976; Garcia-Ramos et al., 1981). This observation indicates that in ionic media, competition for surface sites between the organic and inorganic constituents is dominated by the inorganic components. This result is not surprising considering that the concentration of inorganic constituents is in the millimolar range while the organic ones are at micromolar levels. Similarly, Meyers and Quinn (1971) noted a decrease in organic adsorption when a natural seawater medium was substituted for a sodium chloride medium. It was suggested that these results could also be explained in terms of changes in inorganic components of the medium.

Inorganic particles tested in this study as adsorbents for glucose and glutamic acid were quartz, glass, reagent grade calcite, alumina, illite in the sodium form and

hydroxyapatite. Ion exchange resins used were AG1-X8 and AG3X-4A (anion exchange resins). The anion exchange resins were included because they are known to be good adsorbents. However, it was found that even the adsorption by these adsorbents was limited in the ionic media used (Table 4). Calcite, alumina, illite, and AG1-X8 were tested only in M9 and showed no uptake of either glucose or glutamic acid. Quartz, glass, AG3X-4A, and hydroxyapatite were tested in both M9 and seawater (Table 4). Glass and quartz have no measurable affinity for glucose in M9 or seawater. In seawater, quartz adsorbs 6% of the glutamic acid from a 5 micromolar solution. The probable reason this is not seen in the case of glass is the smaller surface area. The quartz was powdered (0.63 m^2/g), whereas, the glass beads were larger particles (75-150 microns, 0.008 m^2/g). The uptake of glutamic acid on several particle types was studied as a function of time (Figure 12). No change occurs after the first hour up to twenty-four hours. Several experiments were carried on for forty-eight hours; no further adsorption was observed during this time. The adsorption isotherms of glucose and glutamic acid on AG3X-4A and of glutamic acid on hydroxyapatite were examined over the concentration range used in the microcalorimetry assay. The isotherm was found to be described acceptably by a linear function in these ranges (Figure 13). Other workers have found that glutamic acid adsorption to hydroxyapatite is described by a Langmuir isotherm unlike the results in this study (Garcia-Ramos et

Table 4. RESULTS OF ADSORPTION EXPERIMENTS

Particle	Medium	Organic	Ci(1)	Ceq(2)	Q(3)
HA(4)	M9	Glutamic	5.00	4.42	193
HA	Seawater	Glutamic	5.00	3.40	533
HA	M9	Glucose	5.00	5.00	0.0
HA	Seawater	Glucose	5.00	5.00	0.0
AG3X-4A	M9	Glutamic	5.00	3.84	290
AG3X-4A	Seawater	Glutamic	5.00	4.52	120
AG3X-4A	M9	Glucose	5.00	4.40	150
AG3X-4A	Seawater	Glucose	5.00	4.53	117
AG1-X8	M9	Glutamic	5.00	5.00	0.0
Bio Sil A(5)	M9	Glutamic	5.00	5.00	0.0
Illite (6)	M9	Glutamic	5.00	5.00	0.0
Alumina (7)	M9	Glutamic	5.00	5.00	0.0
Calcite (8)	M9	Glutamic	5.00	5.00	0.0
Glass (9)	M9	Glutamic	5.00	5.00	0.0
Glass (9)	Seawater	Glutamic	5.00	5.00	0.0
Glass (9)	M9	Glucose	5.00	5.00	0.0
Glass (9)	Seawater	Glucose	5.00	5.00	0.0
Quartz (10)	M9	Glucose	5.00	5.00	0.0
Quartz (10)	M9	Glutamic	5.00	5.00	0.0
Quartz (11)	M9	Glucose	5.00	5.00	0.0
Quartz (11)	Seawater	Glucose	5.00	5.00	0.0
Quartz (11)	M9	Glutamic	5.00	5.00	0.0
Quartz (11)	Seawater	Glutamic	5.00	4.72	0.22

(1) initial concentration in micromolar
(2) equilibrium concentration in micromolar
(3) amount adsorbed nMole/m^2 (calculated surface area)
(4) Hydroxyapatite
(5) Calbiochem
(6) sodium form
(7) reagent grade Baker
(8) reagent grade Baker
(9) glass beads Sigma
(10) crushed quartz 44-74 microns
(11) powdered quartz

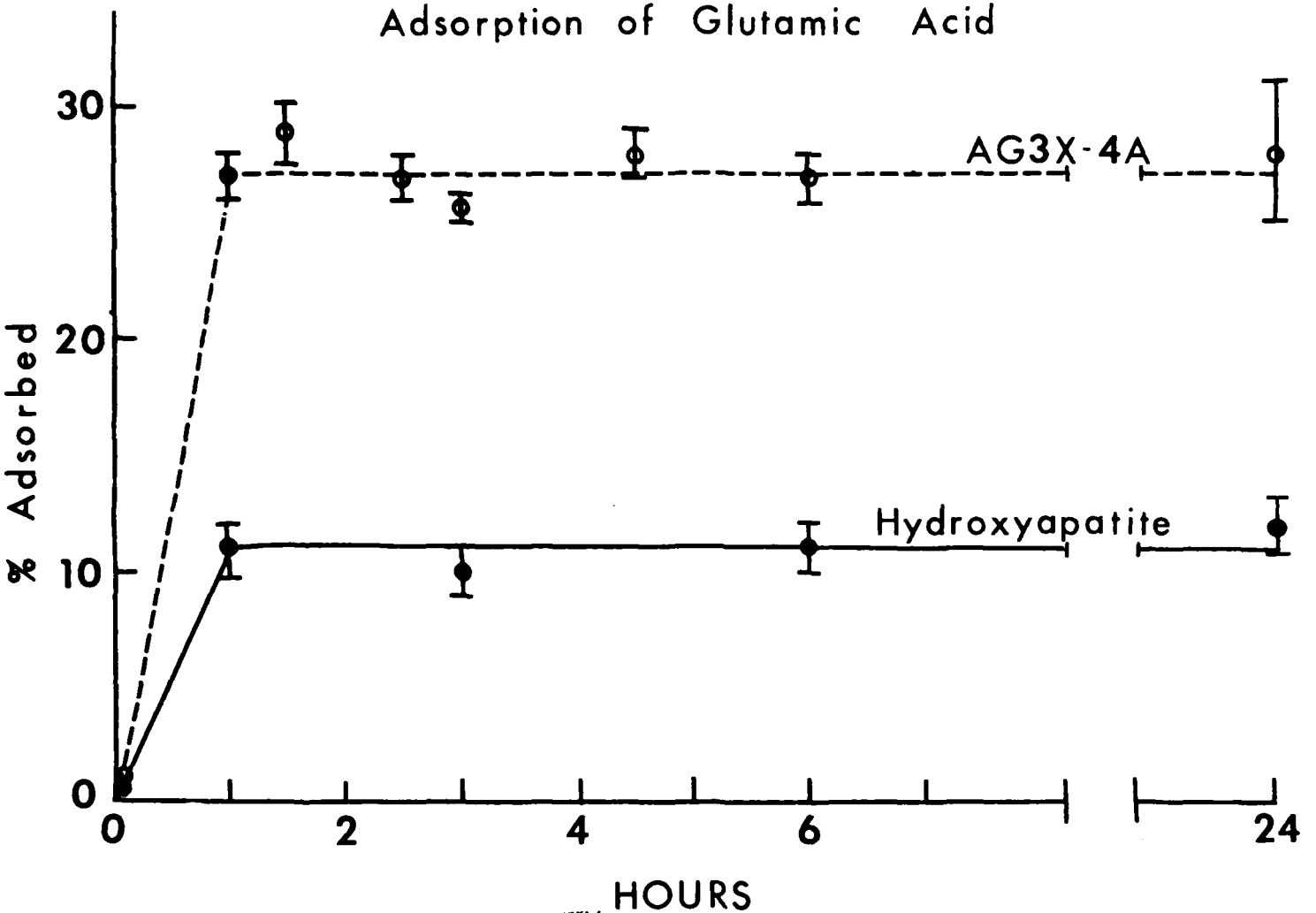


Figure 12. The effect of incubation time on the adsorption of glutamic acid (5×10^{-6} M in M9) on AG3X-4A (anion exchange resin) and hydroxyapatite.

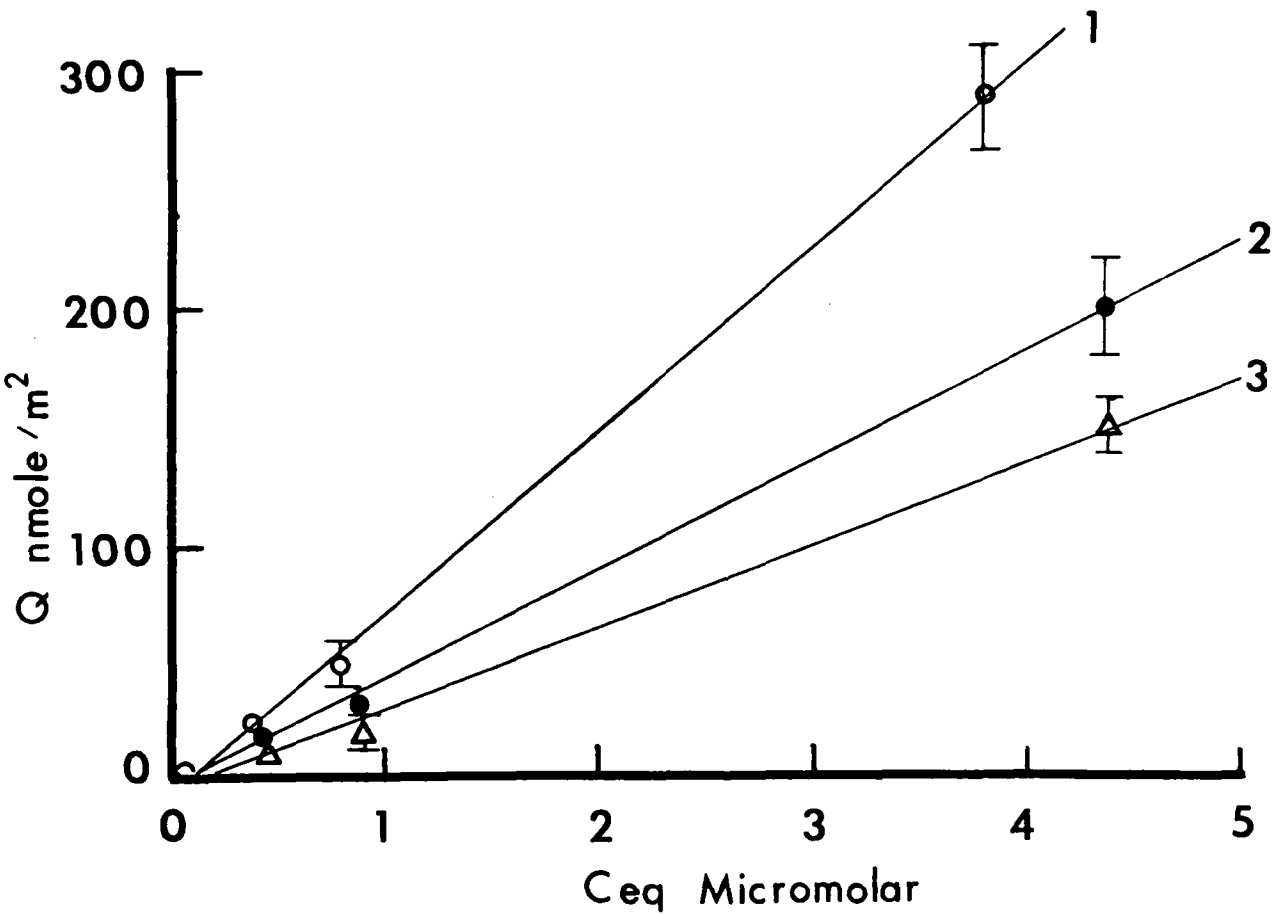


Figure 13. Adsorption of glutamic acid onto AG3X-4A (1), glutamic acid onto hydroxyapatite (2), and glucose onto AG3X-4A (3) as a function of equilibrium concentration in M9.

al.,1981). This discrepancy may be explained by differences in experimental conditions which include suspending medium and concentration of glutamic acid. In this study the glutamic acid was dissolved in either M9 or seawater at concentrations of 0.1 to 5 micromolar. In the study of Garcia-Ramos et al. the glutamic acid was dissolved in distilled water at concentrations ranging from 1.54 to 6.14 millimolar. It is probable that due to the low concentration of organics used in the present study, saturation of the surface was not reached at the concentrations used and that the data falls in the linear region of a curve. Meyers and Quinn (1971) also showed a linear adsorption isotherm of fatty acids on clay at low concentrations of fatty acid.

6.3 Bacterial Attachment Assay

The attachment of the bacteria to hydroxyapatite fits the Langmuir adsorption model. This model describes specific adsorption to an adsorbent. In its linear form the function is described by the equation:

$$C/Q = 1/KN + C/N$$

where C is the equilibrium concentration of adsorbate in solution after adsorption, Q is the amount of the adsorbate associated with the surface, N is the number of adsorption sites, and K is a measure of the strength of the adsorption bond between adsorbate and adsorbent. A plot of Q versus C for adsorption of Vibrio alginolyticus to hydroxyapatite is shown in Figure 14. The linear fit of the data (Figure 15)

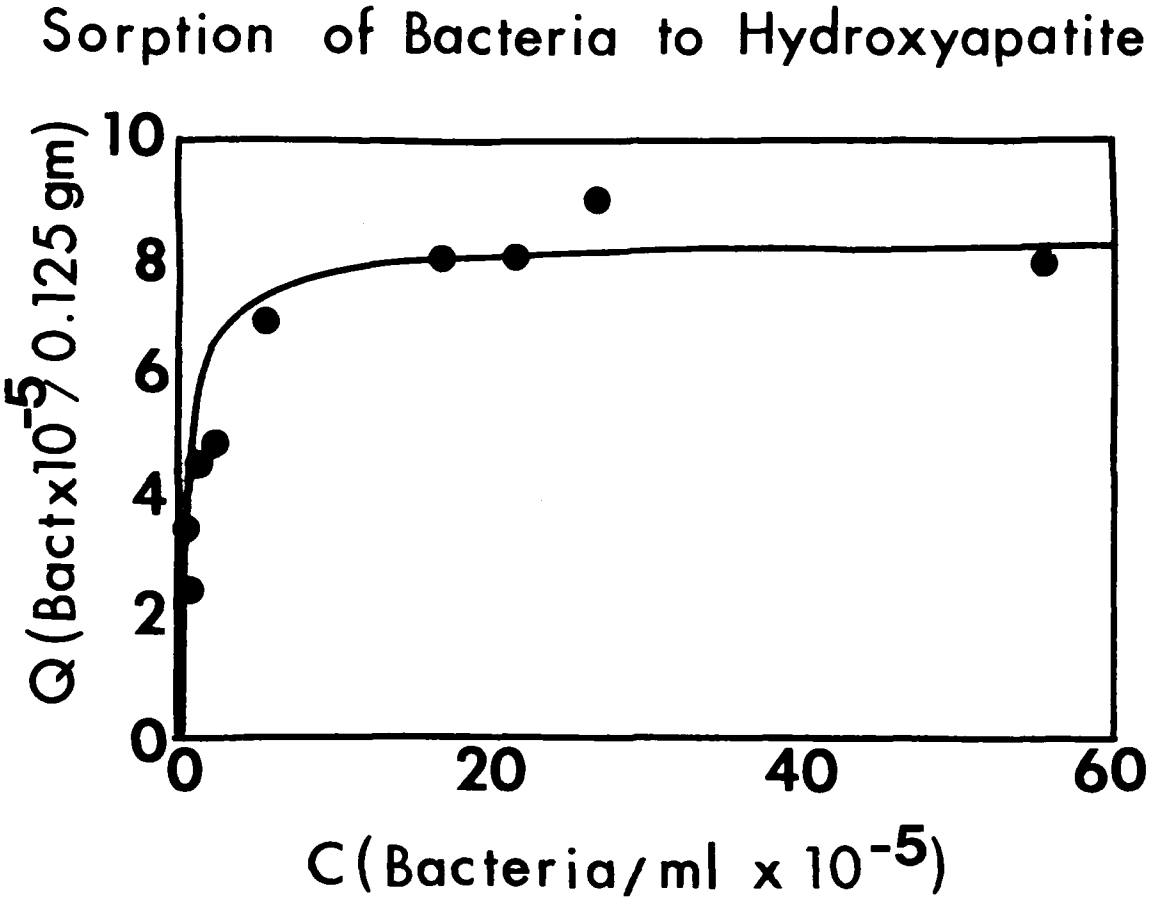


Figure 14. Adsorption of Vibrio alginolyticus to hydroxyapatite in M9 as a function of equilibrium concentration of bacteria.

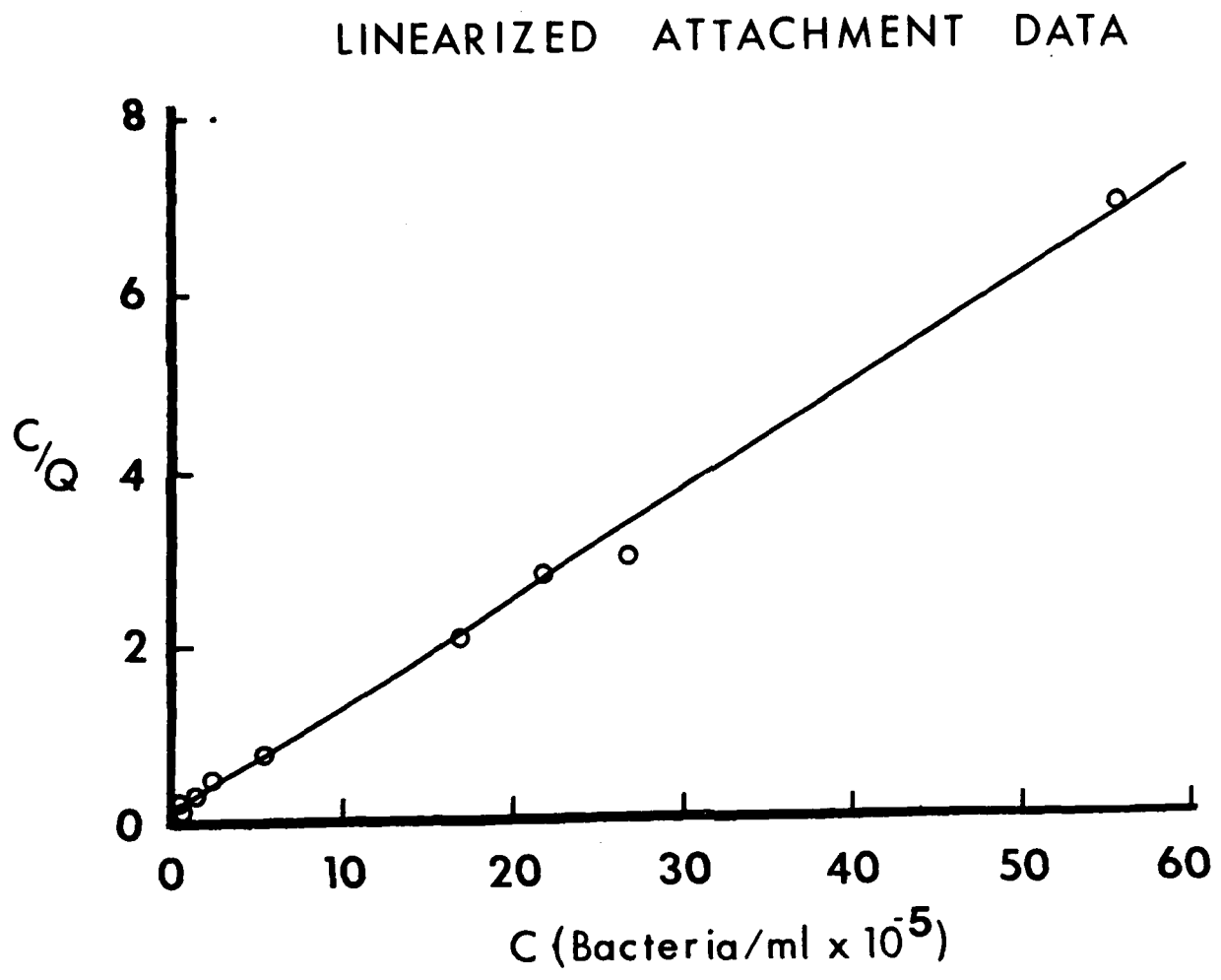


Figure 15. Linearization of the data shown in Figure 16 predicted from the Langmuir adsorption model.

has a correlation coefficient of 0.996, intercept of 0.009 and slope of 0.121 indicating that the data fit the model well. The error of the fit is 0.137 so that the number of bacteria associated with the surface under these fixed conditions can be predicted from the fit to within 1.4×10^4 bacteria. The Langmuir model has been used by other workers t. fit bacterial attachment data and been found to be satisfactory (Gibbons et al., 1976; Clark et al., 1978)

6.4 Surface Area Measurements

Surface areas of the quartz, glass, ion exchange resin, and hydroxyapatite particles used were measured (Table 5). Surface areas calculated on the basis of particle size are also shown for comparison. The calculations assume spherical particles. The calculated and measured surface areas agree well in the cases of glass and quartz but not hydroxyapatite or AG3X-4A. This may be due to microstructure of the particles or, in the case of hydroxyapatite, presence of fine particles that were not removed by the washing procedure. There is some question about the significance of the B.E.T. measured surface area in terms of measurement of surface area that is available for adsorption of bacteria and organic nutrients. It is probable that in the case of glass and quartz, particles without appreciable porosity, the surface area measured by B.E.T. and that available to adsorption of bacteria and

Table 5. SURFACE AREAS (m^2/g)

Sample	Particle size (1)	Calculated (2)	Measured (3)
Glass	75-150	.010	.008
Quartz	44-74	.019	.043
Quartz	powder	----	0.63
Apatite	50-125	.012	43.0
AG3X-4A	100-300	.01	0.23

(1) diameter in micrometers
(2) assuming spheres
(3) measured by krypton B.E.T.

organic nutrients is similar. In the case of hydroxyapatite and the anion exchange resin (a cross-linked organic polymer) there may be a discrepancy between surface available to krypton, small organic molecules and bacteria. The use of this parameter to normalize data on adsorption of bacteria, glucose and glutamic acid may be questioned.

The adsorption of glutamic acid to the particles on a per gram basis, a measured surface area basis and a calculated surface area basis was calculated for comparison of the effect of these various normalization factors (Table 6). As would be expected from the differences in calculated and measured surface areas, these normalization factors affect the apparent relative affinities of these particles for glutamic acid. For example, in M9 the affinity of AG3X-4A and hydroxyapatite for glutamic acid is of the same order of magnitude on a per gram basis and on a calculated surface area basis, while the affinity of AG3X-4A is 233 fold greater when adsorption is normalized on a measured surface area basis. This is due to the similarity in calculated surface areas and the large difference in measured surface areas. In M9 medium, AG3X-4A adsorbs more glutamic acid than hydroxyapatite no matter which normalization factor is used. In seawater, the relative affinities of the particles also change with the different normalization factors. In this medium, the hydroxyapatite adsorbs more glutamic acid than AG3X-4A when normalized on a per gram or calculated square meters basis but less when the data are

Table 6. ADSORPTION OF GLUTAMIC ACID TO PARTICLES IN M9 AND SEAWATER

	Media					
	M9			Seawater		
	Nanomoles adsorbed per:			Nanomoles adsorbed per:		
Substratum	gram	m sq. calc(1)	m sq. meas.(2)	gram	m sq. calc.	m sq. meas.
Apatite	2.32	193	0.05	6.4	533	0.15
AG3X4A	2.90	290	12.6	1.2	120	5.2
Quartz	0	0	0	0.14	---	0.22

(1) surface area in square meters calculated
(2) surface area in square meters measured

normalized to the measured surface area. This again is due to the extremely large difference in the calculated and measured surface area of hydroxyapatite. It is not clear which normalization factor most truly reflects the surface available to bacteria and glutamic acid. For the purposes of the graphics and tables presented in this study, calculated surface areas were selected because it was felt that this number was the best estimation of surface available to bacteria (see section 6.6).

6.5 Metabolism by Bacteria in the Presence of Inorganic Particles

The metabolism of bacteria in the presence of inorganic particles was measured using microcalorimetry and radiorespirometry.

6.5.1 Metabolism of organic nutrients in solution and on surfaces.

Some initial experiments examining the effect of solids at millimolar organic nutrient levels were performed in the Tronac calorimeter (Gordon et al., 1979). The concentration of glucose in these experiments was 16 millimolar. The addition of two grams of either Iceland spar calcite or glass beads (125-150 micron) enhanced heat production during exponential growth (Figure 16). Air was

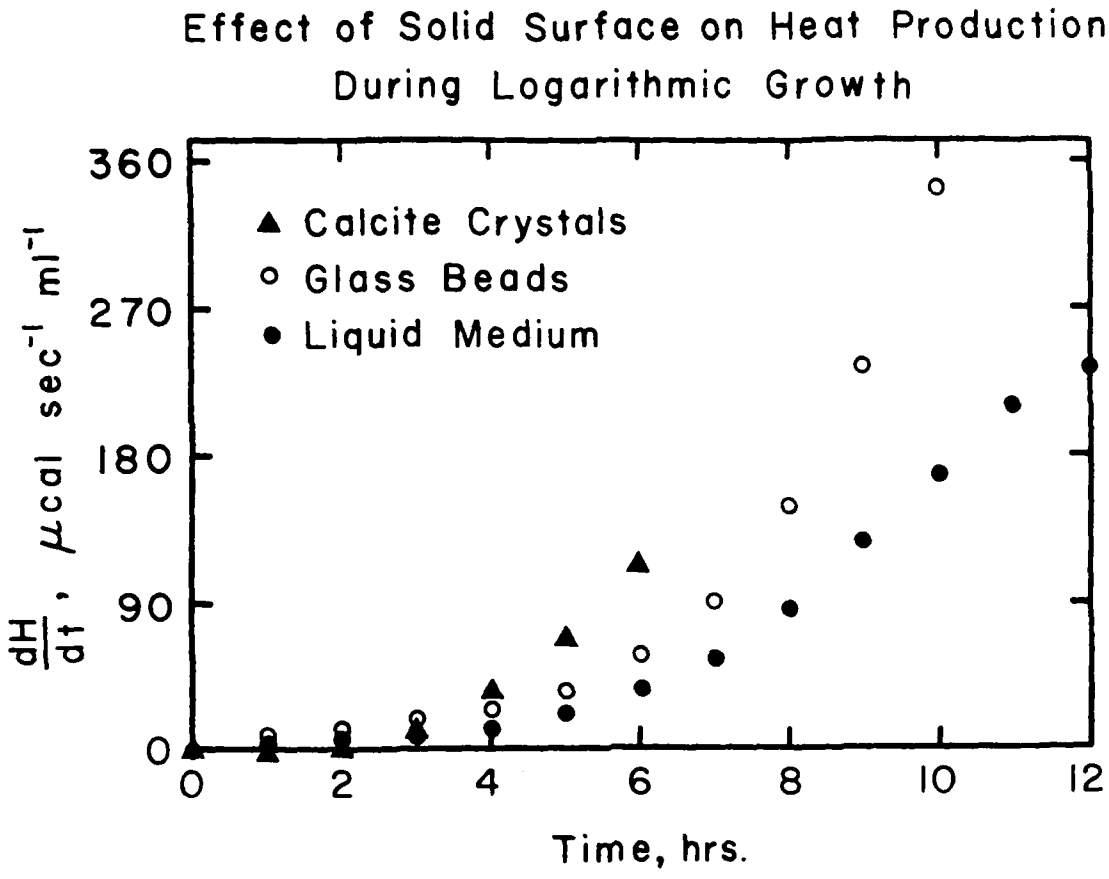


Figure 16. The effect of inorganic particles (glass beads and Iceland spar calcite) on the heat production by Vibrio anguillarum with 16 mM glucose in M9 medium. Air was bubbled through the medium at 5 cc/min.

bubbled through the medium in these experiments. It was subsequently found that this effect disappeared when oxygenation was achieved by purging pure oxygen over the solution rather than bubbling air (Figure 17). It was apparent, then, that the particles were merely increasing aeration efficiency.

Experiments at low substrate concentrations were performed in the LKB calorimetry system and by radiorespirometry. Heat production and respiration by V. alginolyticus at a concentration of $6x10^7$/ml and organic substrate concentration of 5 micromolar were unaffected by the presence of hydroxyapatite particles in the system (Table 7). Metabolic heat production was the same in experiments with and without particles regardless of whether the organic carbon source adsorbed to the particles (as was the case with glutamic acid) or did not (as was the case with glucose). The heat of metabolism was also unaffected by the presence of quartz particles. Radiorespirometry carried out with the same concentrations of bacteria and organic nutrient also showed that the particles did not affect the metabolism of the bacteria.

These results show that the particles have no effect on bacterial metabolism under the experimental conditions. The results also show that within the error of the measurement, glutamic acid is accessible to the bacteria in the system containing hydroxyapatite particles. This suggests either that the adsorbed glutamic acid is available to the bacteria

Table 7. EFFECT OF HYDROXYAPATITE ON THE METABOLISM OF Vibrio alginolyticus.

EXPERIMENT		RESULTS	
		Calorimetry	Respirometry
Conditions		mcal/μmole	nmole respired/μmole
GLUCOSE	No Particles	220 ± 22*	460 ± 22
GLUCOSE	Particles	180 ± 22 (.23)**	480 ± 22 (.52)
GLUTAMIC	No Particles	120 ± 22	440 ± 44
GLUTAMIC	Particles	140 ± 44 (.68)	480 ± 22 (.58)

* Mean standard error

** Student's t-test probability of difference (p value) from experiment without particles

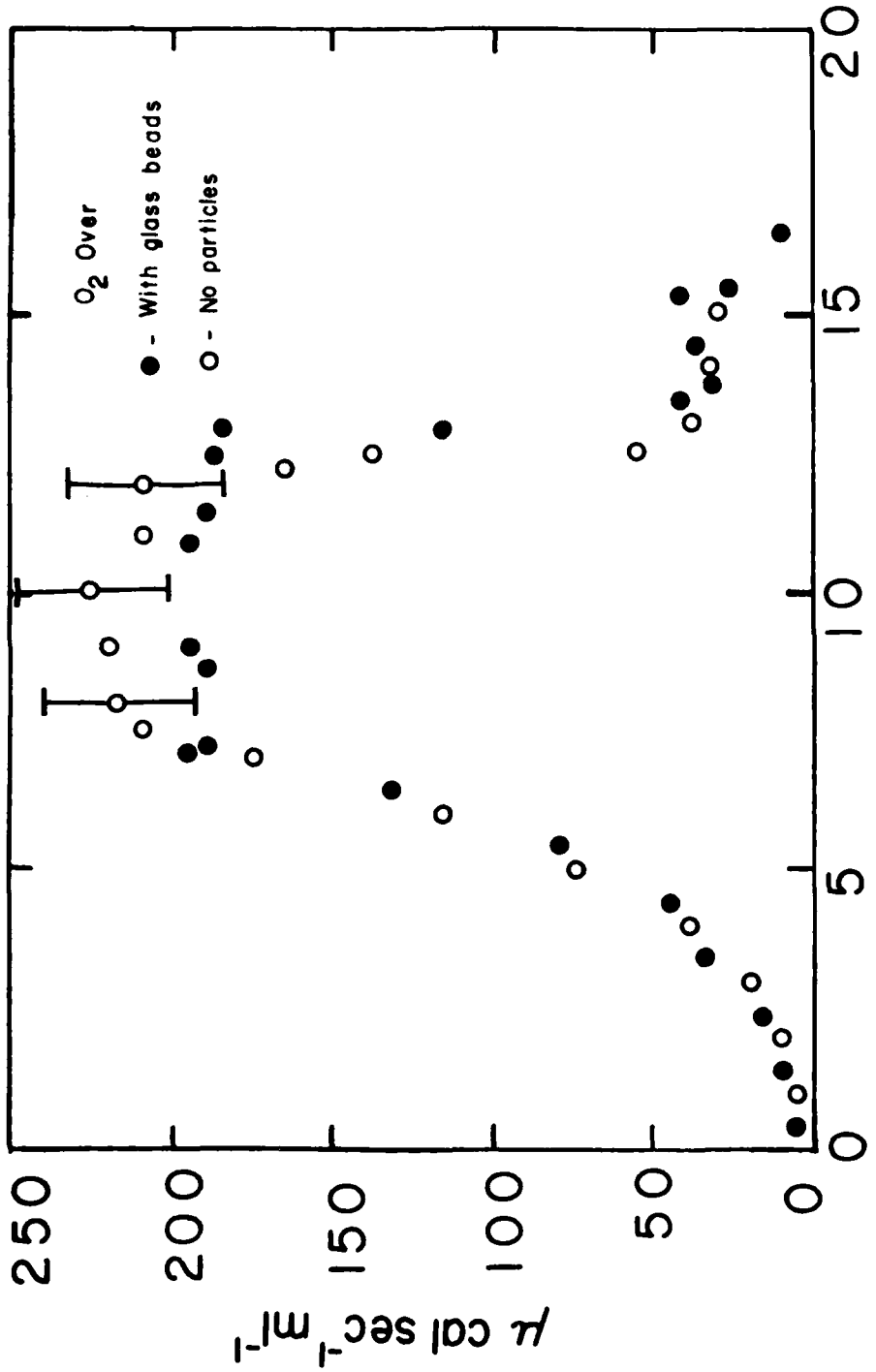


Figure 17. The effect of glass beads on heat production by Vibrio anguillarum in M9 with 16 mM glucose. Oxygen was purged over the medium at 10 cc/min.

or that it becomes available by desorption as the bacteria use the organic nutrient from solution. The data on the adsorption of the bacteria to hydroxyapatite (Figure 14) show only a small fraction of the bacteria are attached to the surface at these concentrations (1-2%). Because of this, the experiments do not show the metabolism of attached bacteria.

6.5.2 Metabolism by attached bacteria

The partitioning of the bacteria between the hydroxyapatite surface and solution is 85% or more only at concentrations below $1*10^{6}$ /ml (Figure 14). The concentration of bacteria in experiments measuring the metabolism of attached bacteria, therefore, had to be on the order of 10^{5} bacteria/ml. The rate of heat production by this number of starved bacteria cannot be detected by the calorimetry system used in this study. No calorimetric data was obtained on attached bacteria. Radiorespirometry and carbon-14 assimilation were the techniques used to study the metabolism of attached bacteria.

The respiratory metabolism of glucose and glutamic acid by bacteria attached to hydroxyapatite surfaces was found to be inhibited in comparison to that of bacteria free in solution (Figure 18 and 19). In addition, assimilation of carbon from glucose was shown to be inhibited (Figure 20). Assimilation of glutamic acid by attached bacteria was not

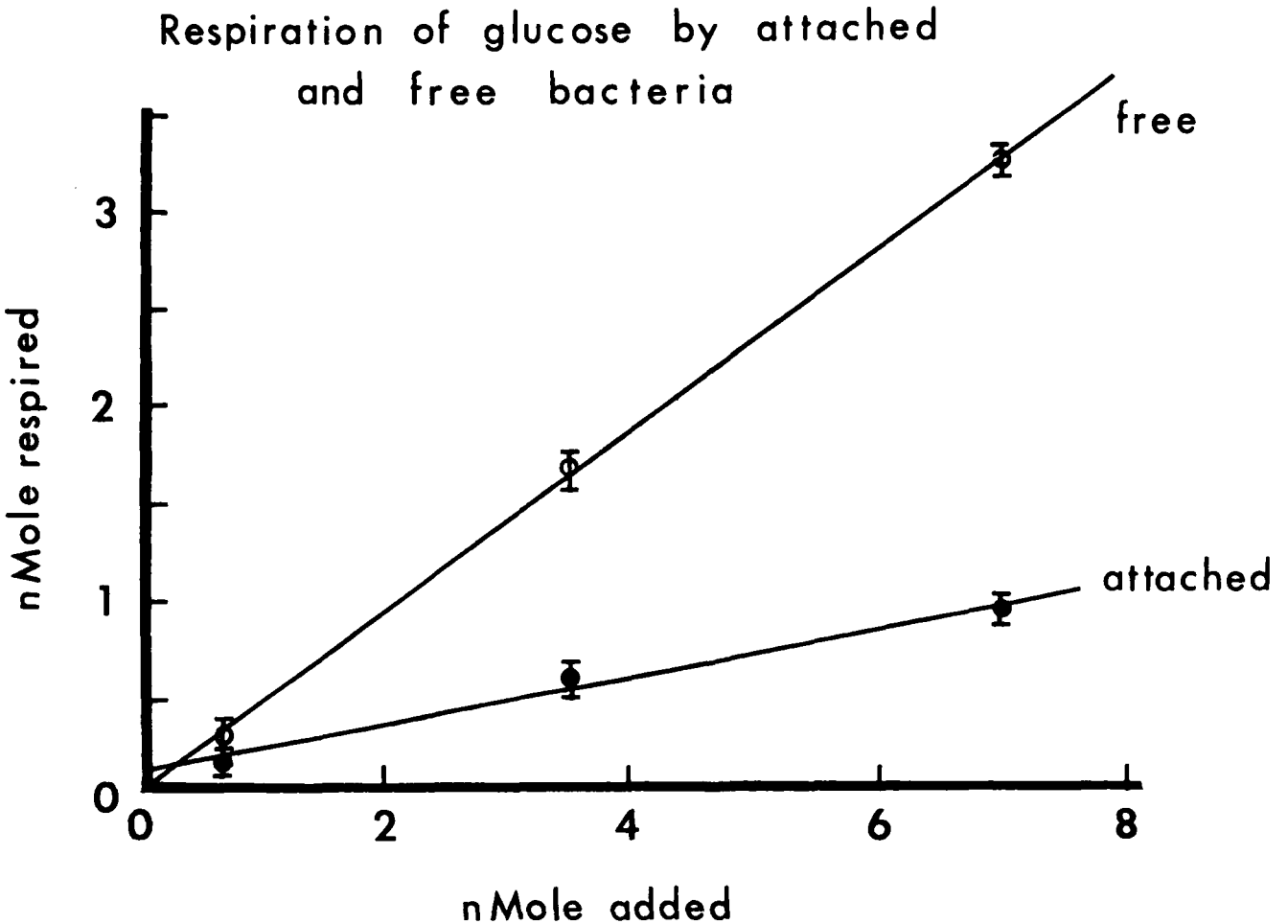


Figure 18. Respiratory metabolism of glucose by Vibrio alginolyticus in suspension compared with those attached to hydroxyapatite particles. The concentration of bacteria was 6×10^5 and the glucose concentration was 5×10^{-6}. The cultures were incubated for twenty-four hours.

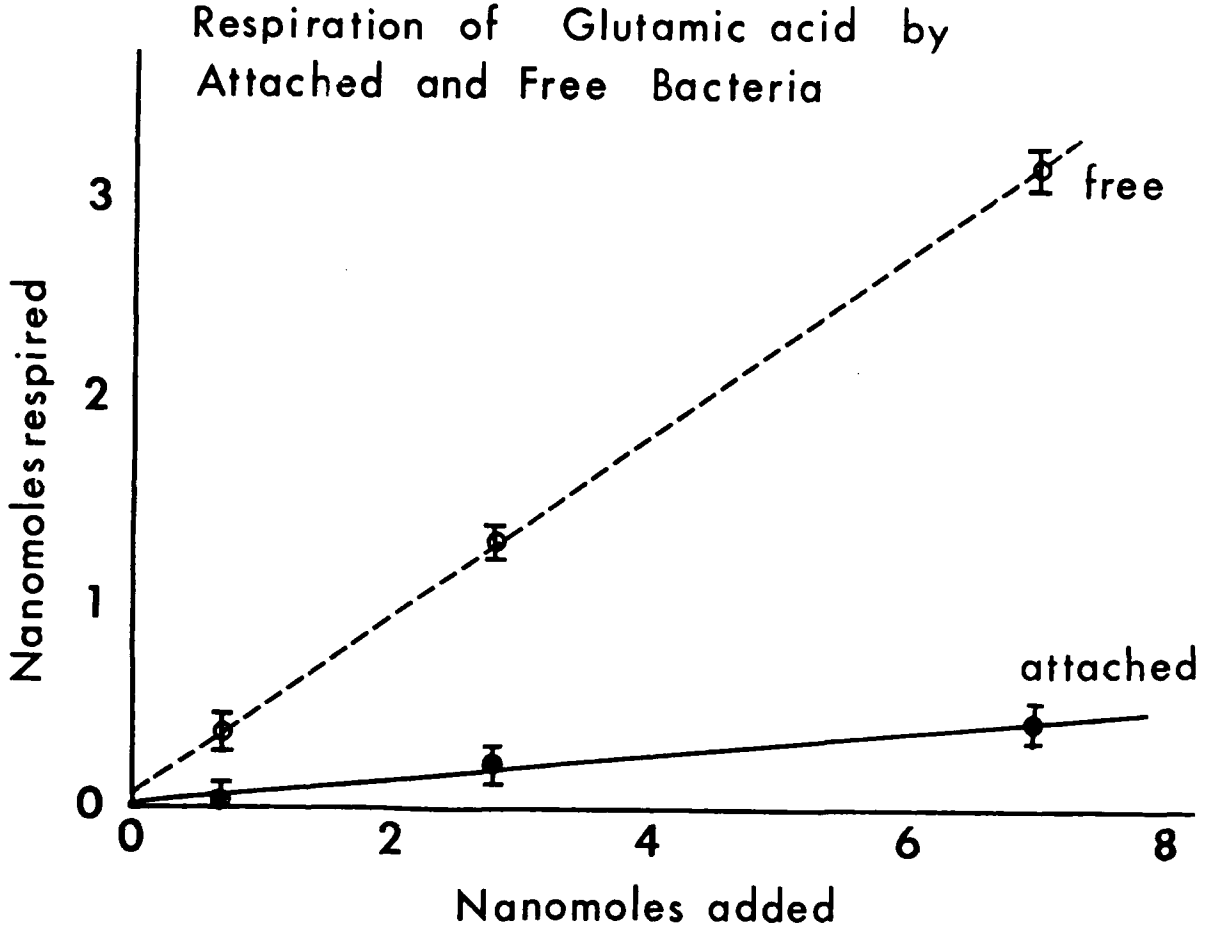


Figure 19. Respiratory metabolism of glutamic acid by Vibrio alginolyticus. Procedures were the same as those for experiments performed with glucose (Figure 18).

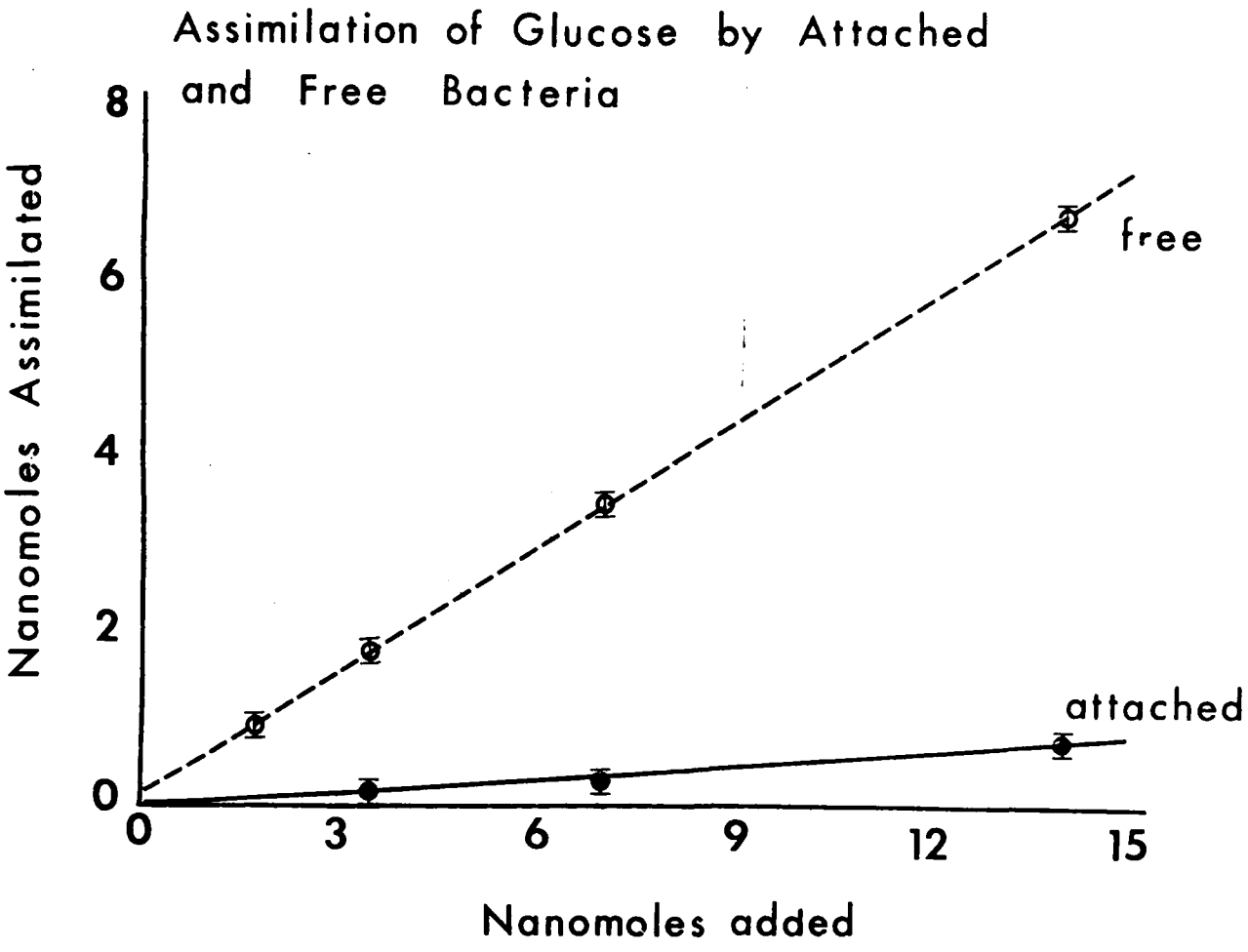


Figure 20. Assimilation of glucose by Vibrio alginolyticus in suspension and on hydroxyapatite surfaces. The concentration of bacteria was 6 x 10^5, the suspensions were incubated for twenty-four hours.

determined due to high number of counts retained by the hydroxyapatite particles. The assimilation of glutamic acid by free bacteria is shown in Figure 21. The carbon dioxide production from glucose by unattached bacteria was 4 times higher than that from attached cells. The assimilation of carbon from glucose by unattached cells was 8 times higher than that from attached cells (Table 8). The ratio of assimilation to respiration was 1:1 for free cells and 1:2 for attached cells. This means that the attached cells had a decreased metabolic efficiency. The respiration of glutamic acid by unattached bacteria was 7 times higher than that from attached cells indicating a decreased activity.

Longterm respiration experiments with the attached bacteria showed that the organisms were indeed active and that they could continue to mineralize the substrates at a low rate until a significant fraction of either substrate was consumed (Figure 22). The metabolic rate of the attached bacteria was lower than that of bacteria in suspension.

If one assumes that the unattached bacteria in the cultures containing particles are equally as active as those in cultures without particles, 1/2 of the respiratory activity in glucose cultures containing particles and all of the respiration in the glutamic acid cultures containing particles could be accounted for by the free cells. The percent of free cells in these experiments calculated from the results in section 6.3 is 13. If the respiration data are corrected for the contribution of unattached

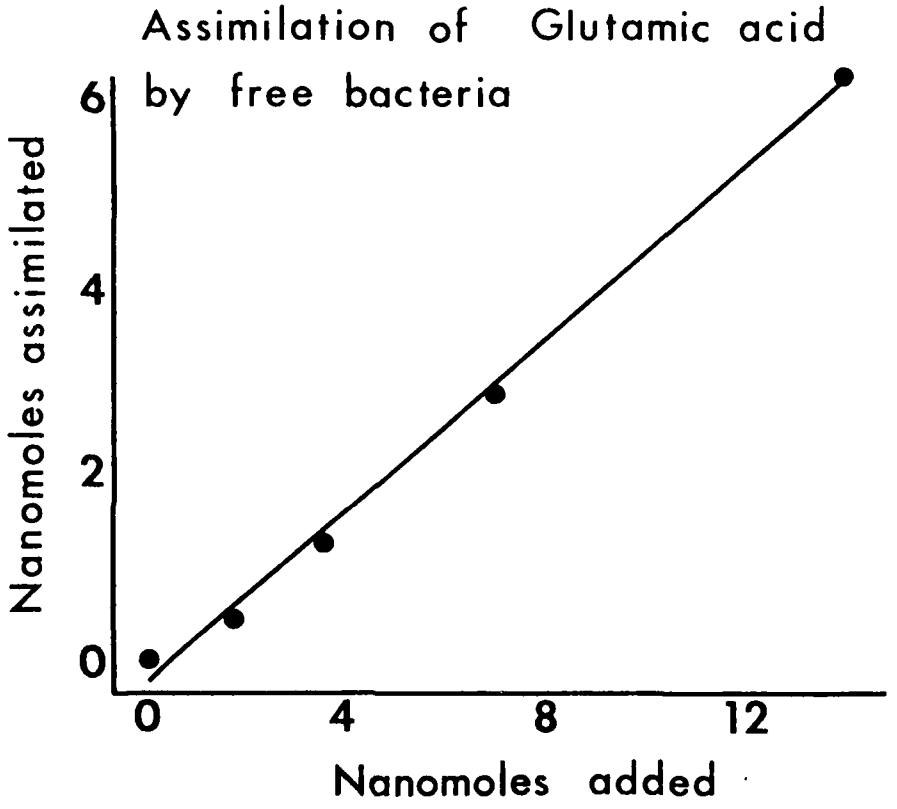


Figure 21. Assimilation of glutamic acid by Vibrio alginolyticus (6×10^5/ml). The suspension was incubated for twenty-four hours.

Table 8. THE RESPIRATION AND ASSIMILATION BY ATTACHED AND FREE BACTERIA

	GLUCOSE	
	RESPIRATION %	ASSIMILATION %
FREE	47 ± 5	48 ± 5
ATTACHED	12 ± 6	6 ± 7
	GLUTAMIC ACID	
FREE	44 ± 1	47 ± 5
ATTACHED	6 ± 3	---

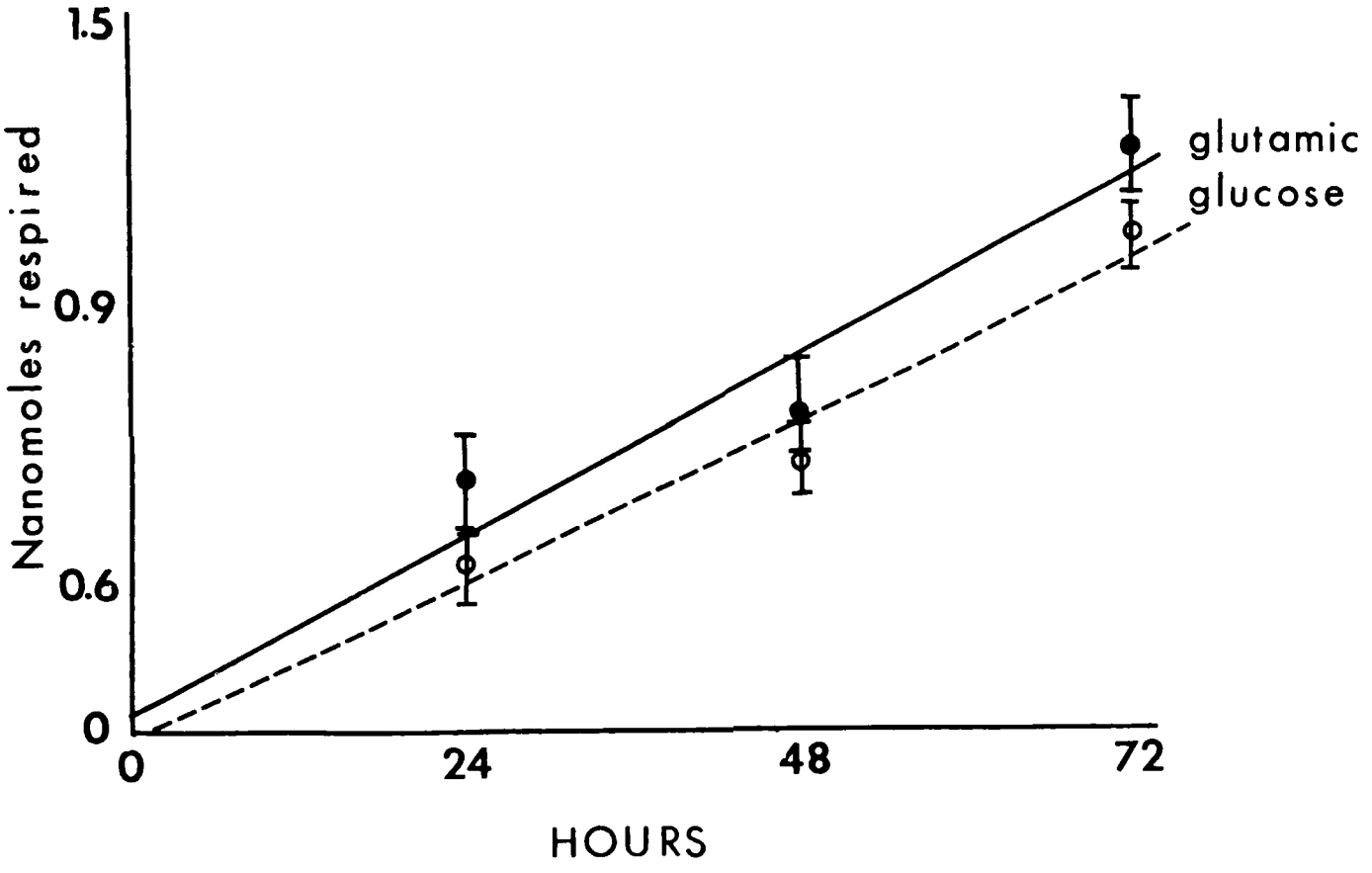


Figure 22. Respiratory metabolism of glucose and glutamic acid by Vibrio alginolyticus attached to hydroxyapatite surfaces over a period of seventy-two hours. The concentration of organic nutrient was 5×10^{-6} M in M9.

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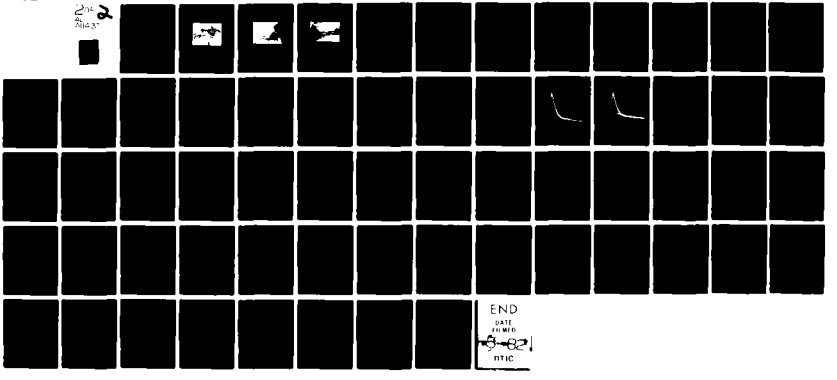

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bacteria, it appears that the attached cells are even less active than the uncorrected data indicate.

6.6 Scanning Electron Microscopy

Micrographs were made of bacteria attached to glass, quartz, and hydroxyapatite surfaces. The bacteria attach to each of these surfaces from M9 medium. While bacteria are clearly discernible on the glass (Figure 23) and the quartz (Figure 24), it is more difficult to observe the bacteria on the hydroxyapatite surface (Figure 25). This is due to the many irregularities on the hydroxyapatite surface, many of which look much like bacteria.

The hydroxyapatite surface appears to be almost sponge-like. This would account for the large difference in calculated and observed surface area of the material (section 6.4).

No extracellular material or flagella were observed associated with any single cells on any of the specimens. Some capsular material appears to be associated with the colony on the glass surface (Figure 23). It is possible that these structures were damaged during the fixation procedure.

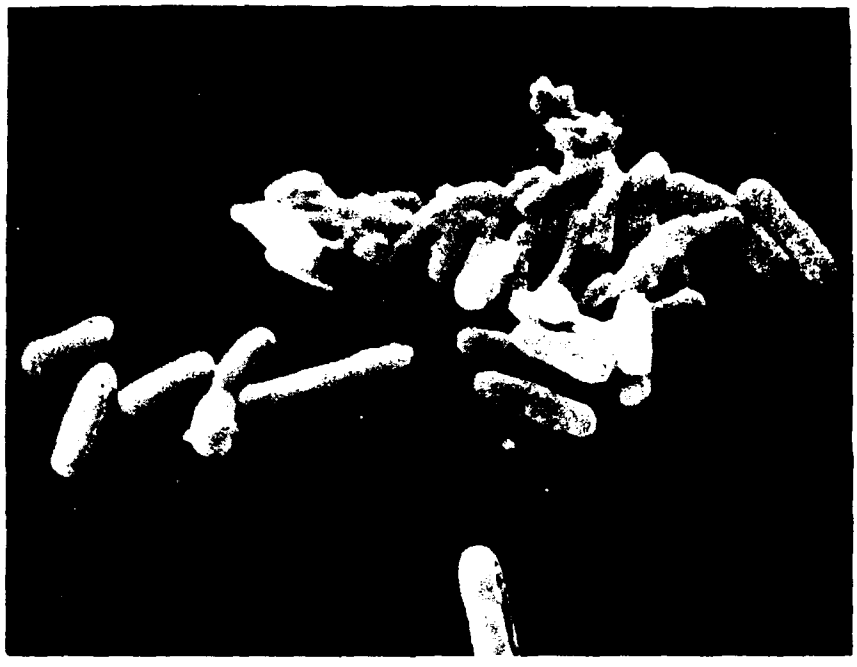

Figure 23. Scanning electron micrograph of Vibrio alginolyticus attached to glass. Magnification 11.5 K.

Figure 24. Scanning electron micrograph of Vibrio alginolyticus attached to quartz. Magnification 8.3 K.

Figure 25. Scanning electron micrograph of Vibrio alginolyticus attached to hydroxyapatite. Magnification 5.9 K.

7. DISCUSSION

The results of the present study do not support the initial supposition that the activity of marine bacteria is stimulated by the presence of inorganic surfaces. Bacterial activity was not enhanced at high (millimolar) concentrations of glucose (Figure 17) or at low concentrations (micromolar) of glucose or glutamic acid. Bacterial activity was not enhanced regardless of whether the bacteria, the organic nutrient or both were associated with the surface. In fact, the activity of the attached bacteria was diminished in comparison with free bacteria.

There are some important differences between the experimental systems employed in this study and those used by some of the workers who initially observed the "surface effect" and who attributed it to increased availability of organic nutrients due to their adsorption on surfaces. In the now classic work by Zobell (1943), respiration and plate count measurements showed that bacteria multiply in seawater stored in clean glass bottles and this effect is related to the glass surface area exposed to the seawater. This effect has been observed by other workers (Waksman and Carey, 1935). The effect is variable from sample to sample of seawater. The variation was attributed to variations in

organic content and bacterial populations in the samples. In natural seawater a large proportion of the total organic carbon is uncharacterized material (Williams, 1975) which is thought to be relatively resistant to biodegradation (Skopintsev, 1981). Some of these materials may be expected to adsorb to surfaces like glass. For example, fulvic acid adsorbs to quartz and calcite (Carter,1978). As is the case with oil droplets, the availability of these organic compounds to microorganisms could be limited by the exposed surface area of the organic material (Marshall, 1976; Rosenberg, 1981). The exposed surface area of the organic material could be increased by adsorption onto glass. Kriss (1963) proposed an explanation for Zobell's results as an alternative to the theory that organic nutrients are concentrated by adsorption. He proposed that refractory material from seawater is rendered biodegradable by qualitative changes occurring at the interface. In particular, he claims that proteinaceous material in seawater is rendered more susceptible to attack by bacteria due to denaturation on the surface. In cases where such refractory organic material is the organic nutrient, relatively low surface areas, as were used by Zobell, and relatively inert surfaces, such as glass, could reasonably be expected to exert an influence on microbial growth.

The situation in natural seawater is so complex that it is difficult to sort out the factors contributing to changes in microbial activity. As observed in the present

study, inorganic nutrients can play an important role in determining the rates of heterotrophic microbial activity in seawater (section 6.1.2). It is well known that inorganic nutrients such as ammonium and phosphate can adsorb to surfaces (de Kanal and Morse, 1978; Eaton and Grant, 1978). This may create a microenvironment enriched in these nutrients at the surface. Because of the complexity of the natural system a controlled, well defined laboratory system was chosen for the present study in which inorganic nutrient limitation could be removed.

Zobell (1943) did not observe any adsorption of low molecular weight, soluble organic material to glass surfaces. No adsorption of glucose or glutamic acid was observed in the present study when similar surface areas were used. However, Heukelikian and Heller (1940) observed an enhancement of bacterial growth by glass surfaces in a laboratory system when glucose was used as energy source and peptone as nitrogen source. The phosphate concentration was quite low (50 ppm) in the studies of Heukelikian and Heller and may have been a colimiting factor to bacterial growth along with glucose. Also, cleaning procedures were poorly defined and contamination by phosphates from detergents may have influenced the results. Peroni and Laverello (1980) have shown that cleaning procedures can lead to contamination arising from the detergents used and cause increased bacterial growth due to contamination of laboratory glassware. Another possible explanation of Heukelikian and

Heller's observation is the adsorption of incompletely hydrolyzed peptide components of peptone on the glass surface leading to the ability of the organisms to grow at lower substrate levels in the presence of the surface. Zobell, however, did not note any adsorption of peptone to glass in his experimental system. The method of analysis of bacterial growth in Heukelikian and Heller's experiments was plate counts after shaking to remove the bacteria associated with the surface. It is quite possible that clumping or dividing cells may have been separated by this procedure leading to apparently higher cell concentrations in flasks containing particles. No suitable control for this possibility was reported. To my knowledge no independent studies have reproduced the work of Heukelikian and Heller.

The results of the present study concerning the metabolic activity of attached bacteria are consistent with the observations of Hattori and Furasaka (1960, 1961) who found that oxygen consumption by E. coli and A. agile was inhibited when the cells were attached to an anion exchange resin. These workers attributed the effect to calculated pH differences between the surface microenvironment and the bulk solution. The results of the present study may also be interpreted as being due to differences in conditions between the surface and the solution. Differences in conditions at the interface could be due to many factors including the availability of inorganic nutrients, pH, availability of oxygen, and the proximity of the bacteria to one another.

Certain attributes of the bacteria may be altered by attachment to a solid surface such as motility and exposed cell surface area which would be decreased. On the basis of the available data it is not possible to conclude which of these factors, if any, are responsible for the diminished activity of bacteria attached to surfaces in the present study. It does not appear that concentration of the organic nutrient on the surface alleviates the inhibition observed. This suggests that the inhibition is not due to a decrease or increase in the concentration of the carbon source at the surface. One would not expect any decrease in available phosphate or calcium on the surface of hydroxyapatite, a calcium phosphate mineral. In addition, since glutamic acid does not alleviate the inhibition of respiration and is a nitrogen source which adsorbs to the surface, one would not expect that nitrogen availability is the controlling factor. If the observed decrease in bacterial activity at the interface is due to changed growth conditions there, then the most likely factors appear to be oxygen availability, trace metal availability or some parameter such as pH.

To the extent that they are representative of natural conditions, the results of this study do not support the hypothesis that attached bacteria are mainly responsible for turnover of labile organic nutrients in natural waters. The postulate that bacteria are more efficient at scavenging organic nutrients when they are attached to surfaces does not hold in this model system. In fact, bacterial activity

was inhibited when the organisms were attached to a surface. The question remains then: why was this bacterium and many others physiologcally similar to it found colonizing inorganic surfaces in Biscayne Bay (Gerchakov et al. 1976)? This bacterium has efficient, constitutive capabilities for capturing and metabolizing glucose and glutamic acid from solution. This observation indicates that the bacterium uses these substrates in nature. If the bacterium is indeed using these substrates on the surface in nature, and the enrichment of these organic nutrients by adsorption on the surface does not contribute to enhanced bacterial activity, then it may be that the surfaces do not serve primarily as areas of concentration of organic nutrients from the solution as much as they serve as sources of these nutrients. Surfaces may serve as centers of production of biomass and associated metabolites in relatively barren, oligotrophic waters. As was previously mentioned, many algae are observed on inorganic surfaces exposed to natural waters (Marszalek et al. 1979; Seiburth, 1975). It is known that Vibrio alginolyticus is attracted to algal extracellular products (Sjoblad and Mitchell, 1979). However, if one hypothesizes that bacteria come to the surface for food produced there by other organisms, one must answer another question which arises from this hypothesis: why are bacteria the first colonizers of inorganic surfaces exposed to natural waters? (Marszalek et al., 1979; Marshall, 1976). The answer to this question may be that bacteria of

different physiological types attach at different times during the fouling process. There appears to be a succession of bacterial types within the early colonization by bacteria (Marshall, 1976). The first bacteria to colonize a surface may indeed be those attracted to material adsorbed from natural water. Later, other bacteria may be attracted by metabolites produced at the surface. The bacteria used in this study were isolated after the 18th day of exposure and so may have been attracted by algal exudates; algae colonize the surface after several days (Marshall, 1971; Corpe, 1973). It would be interesting to compare the physiology of early and late colonizing bacteria in terms of their affinity for the material which adsorbs to glass from seawater and for surfaces covered with this material.

Assuming that bacteria must be attracted to a surface to attach to it may be overly anthropomorphic. Must the bacteria actively seek the surface or are they passively adsorbed? Bacteria clearly attach to surfaces which do not stimulate their activity (Hattori and Furasaka, 1960, 1961; Fletcher, 1979B; this study). However there is also an active process involved in attachment; this is the secretion of polysaccharide holdfast material to anchor the cells (Fletcher and Floodgate, 1973; Corpe, 1970, 1980; Fletcher, 1980). These two observations are not mutually exclusive. There are two phases to bacterial attachment (Marshall et al., 1971); these are an initial phase in which bacteria behave essentially like colloidal particles (passive) and a

subsequent phase during which the bacteria are thought to actively secrete exopolymeric holdfast material (active). Bacteria which have adapted to live on surfaces in natural waters may have evolved "stickiness" to enhance their chances of adhering to a surface during the passive phase of attachment. A sticky bacterium would have selective advantages over nonsticky ones if indeed attachment is generally advantageous. Sticky bacteria would have a high affinity for surfaces during the initial stages of attachment as well as the capability of becoming firmly attached by active secretion of exopolymers. If some bacteria adhered to a surface that did not present a favorable environment then the stickiness would become a disadvantage. As long as attachment is generally advantageous then overall, stickiness would be a positive factor for natural selection. If such random processes control attachment, then bacteria may be the early colonizers of surfaces merely because stickiness has been selected for and consequently the mechanism of initial adhesion is very effective.

It is possible that the shift-down in metabolic rate due to attachment to hydroxyapatite surfaces is not due to a change in growth conditions at the interface. If a bacterium in nature were to attach to a surface which was not itself usable as a nutrient source it might be advantageous for the bacterium to slow its metabolic rate. This lowered metabolic rate could be a survival mechanism in

nutrient poor waters while attachment to a surface could provide a means of transport to a richer environment. Particles will be transported to the sediments while larger surfaces will become colonized by primary producers. The observation by Dawson et al. (1981) that starved bacteria attach to surfaces in significantly higher numbers than exponentially growing cells may be interpreted as supporting this idea. Starved bacteria could, by adhering to surfaces be transported from their current environment to another, hopefully richer, one.

In the complex system of the natural microfouling community it appears there is room for particular instances that support various mechanistic hypotheses about why heterotrophic bacteria attach to inorganic surfaces. In fact, a single mechanistic hypothesis intended for all bacteria and all surfaces clearly will be inadequate

8. SUGGESTIONS FOR FUTURE RESEARCH

Further research is clearly needed before we will be able to understand exactly what the advantages of bacterial attachment to inorganic surfces are in marine environments (if indeed there are any) and what controls the activity of attached bacteria. At this point, it is not possible to identify the organic components of seawater which contribute to the "surface effect". The present study indicates that the surface effect is not solely due to the attachment of bacteria to a surface or to the adsorption of monomeric organic nutrients on a surface.

If one is to be able to conclusively demonstrate the mechanism of the "surface effect" then it must be reconstructed in the laboratory with surfaces, nutrients, heterotrophic bacteria and possibly other chemical or biological components from seawater so that the critical factors may be identified. There are several possible approaches to this task. These are to reconstruct the effect using defined reagents, as was the approach in the present study, or to fractionate the components from seawater and reconstruct the effect by recombination of these. Using the first approach one could continue in the manner used in this study, extending the system to more complex organic

nutrients which have higher surface activity and which may require exoenzymes for degradation. In addition, the system could be expanded to include mixed cultures of periphytic algae and bacteria in order to study nutrient transfer between these organisms. The second approach, although it is somewhat of a "black box" method, might be helpful for identifying the general class(es) of organic nutrient which is the most important contributor to the "surface effect".

Further studies of the activity of bacteria attached to inorganic surfaces may help in providing an answer to the question of why Vibrio alginolyticus has a lowered metabolic rate when it is attached to hydroxyapatite.

9. APPENDICES

9.1 Appendix A- Bacterial Calorimetry

9.1.1 Automated data acquisition

A data collection system was constructed so that, if desired, data from calorimetry experiments could be gathered on paper punch tape on a teletype and loaded into Hewlett-Packard 9830 mini-computer for plotting and data analysis. Schematic diagrams of the data flow for the Tronac and LKB calorimeters are shown in Figures 26 and 27. Data points from the Tronac and LKB systems were collected every 100 and 4.2 seconds respectively. An example of the plot derived from the Tronac is shown in Figure 28. A plot of an LKB experiment is shown in Figure 29. Programs were written to integrate thermograms and to coplot replicate experiments as shown in Figure 30 or to compare experiments performed under different conditions as shown in Figure 31. Different experiments could be plotted with the experimental errors for assessment of regions of significant differences (Figure 31). By the end of this project an in line mini-computer (Commadore Business Machines Model 32N) was added to the system so that data was read directly into the computer for analysis and storage on floppy disk. A schematic of this system is shown in Figure 27.

9.1.2 Discussion of microcalorimetry as a tool for studying metabolism by marine bacteria.

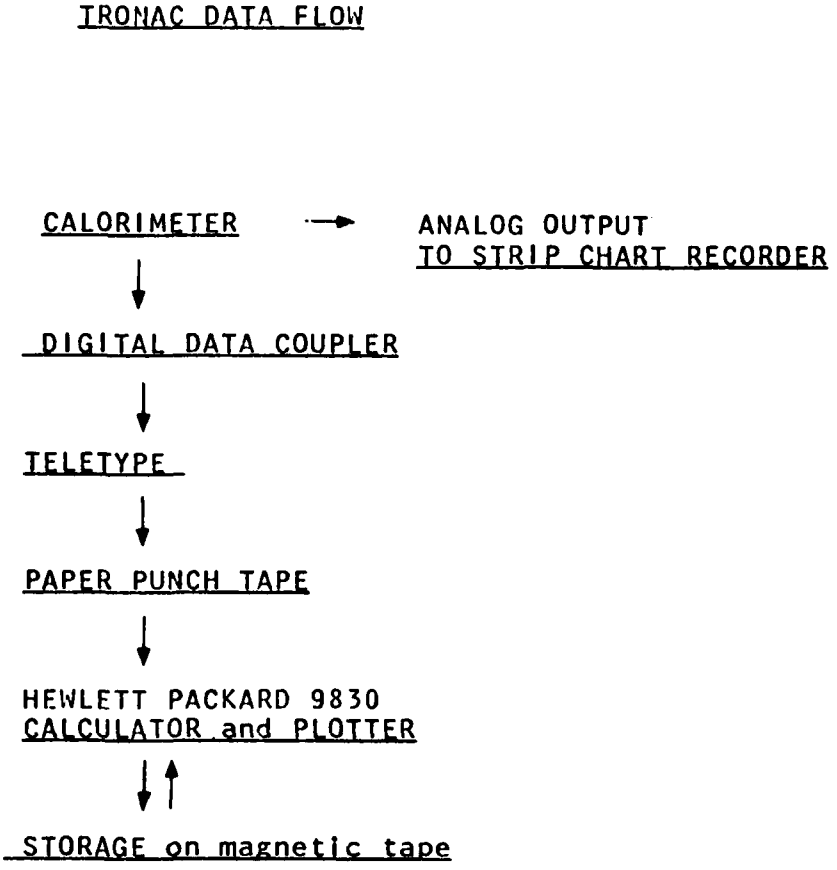

Figure 26. Flow diagram for the data from the Tronac calorimetry experiments.

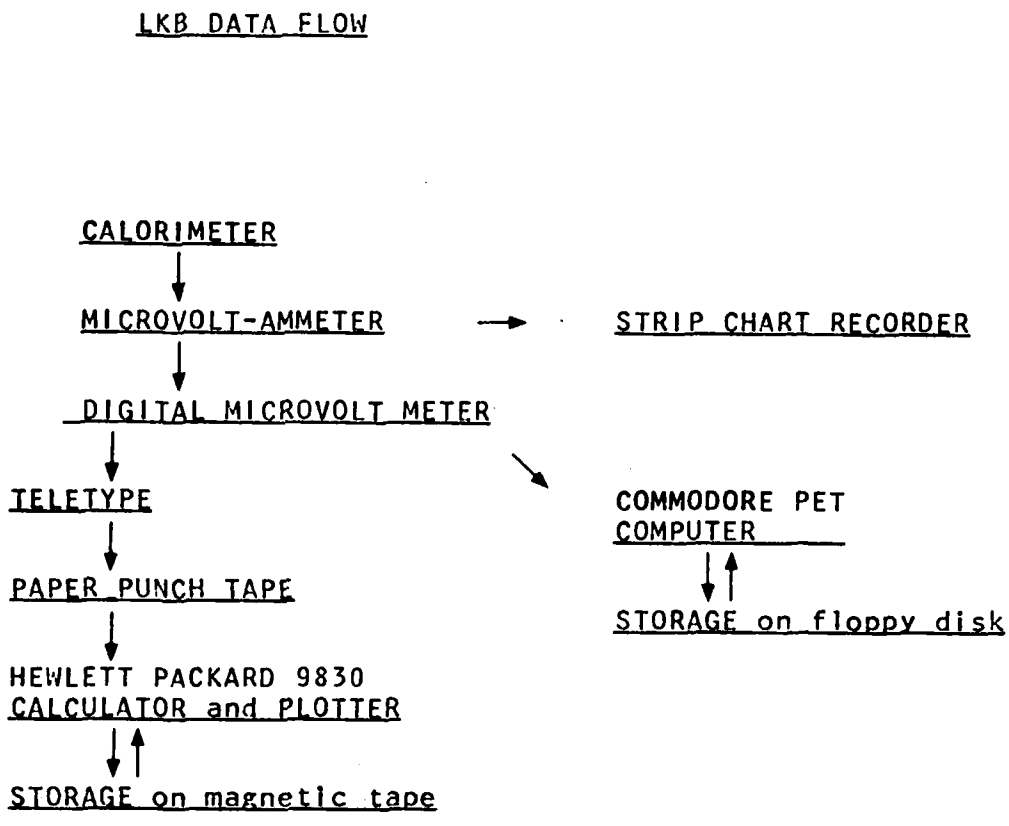

Figure 27. Flow diagram for the data from the LKB calorimetry experiments.

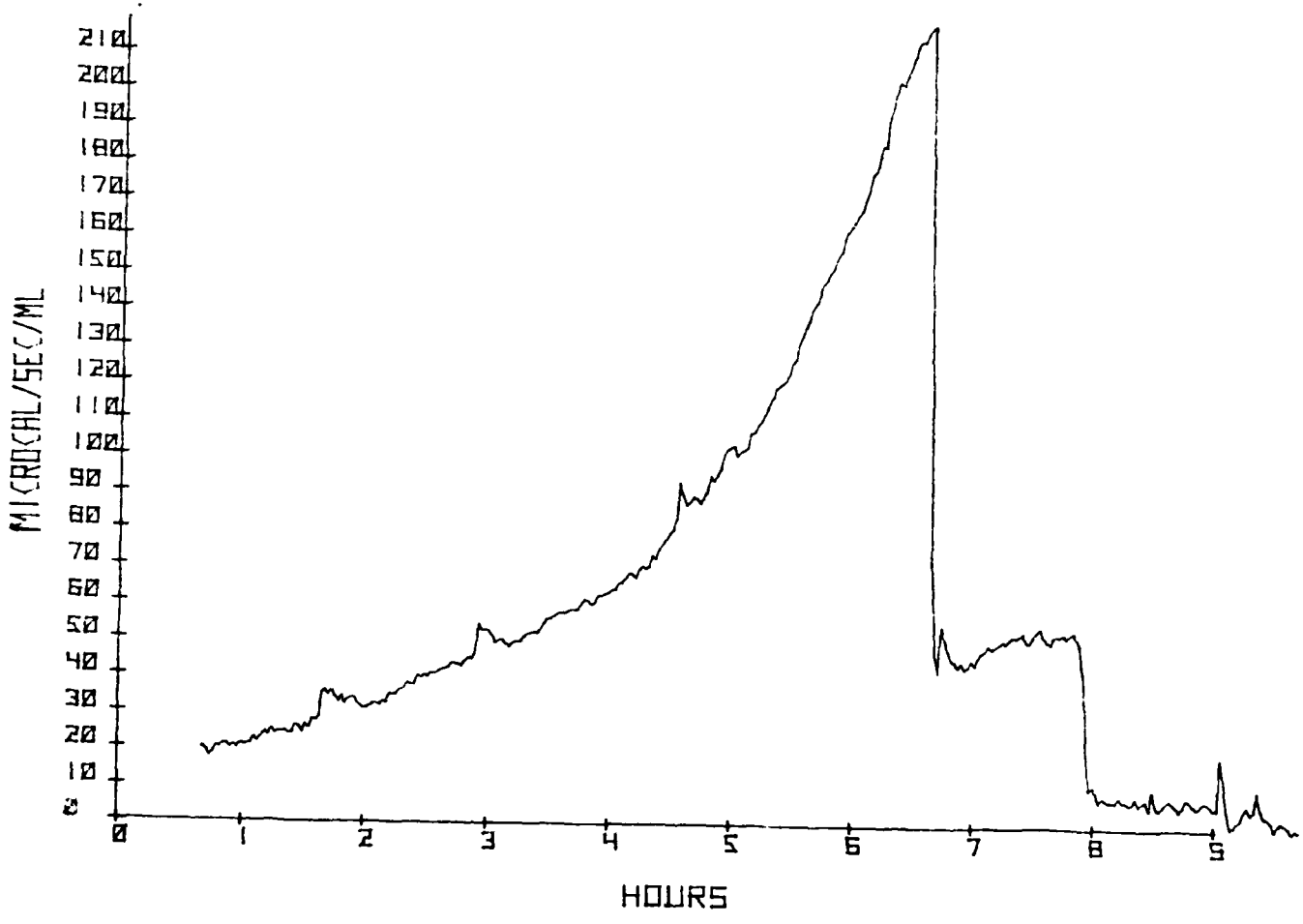

Figure 28. Sample thermogram derived from the automated data processing system for the Tronac calorimetry system.

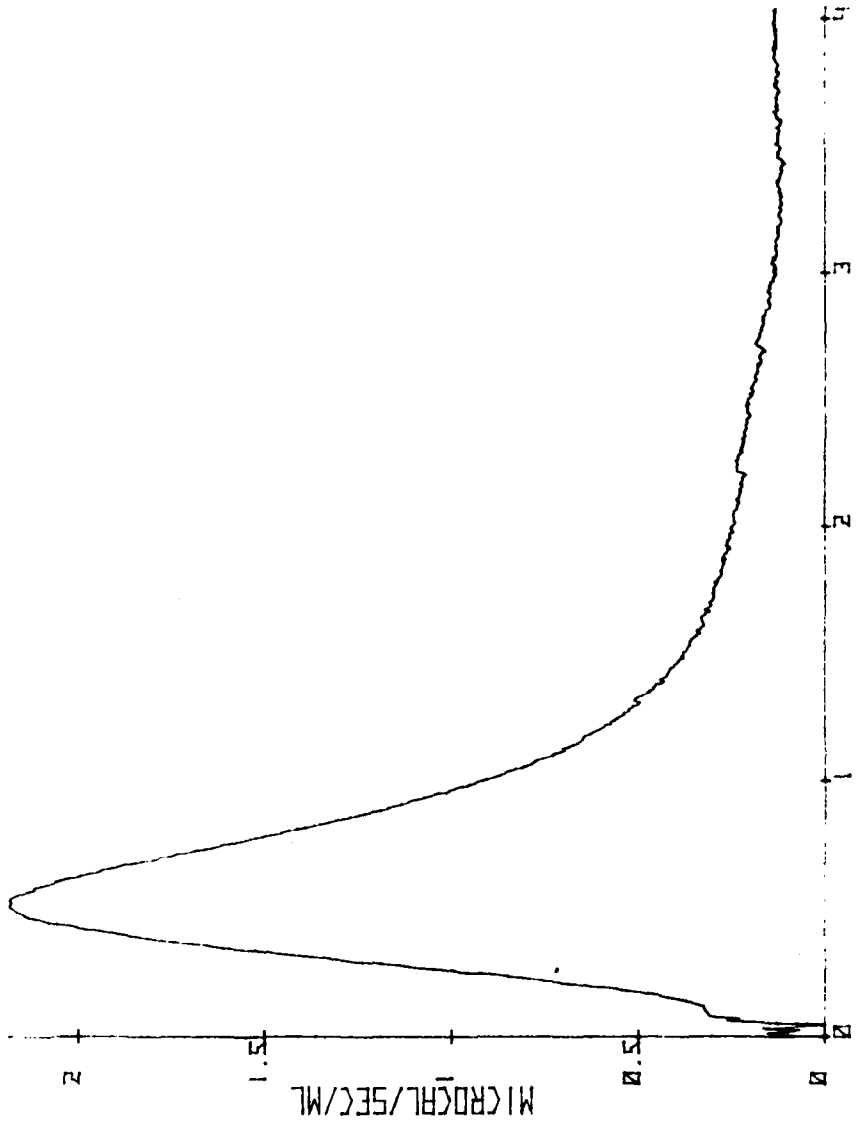

Figure 29. Sample thermogram derived from the automated data processing system for the LKB calorimetry system.

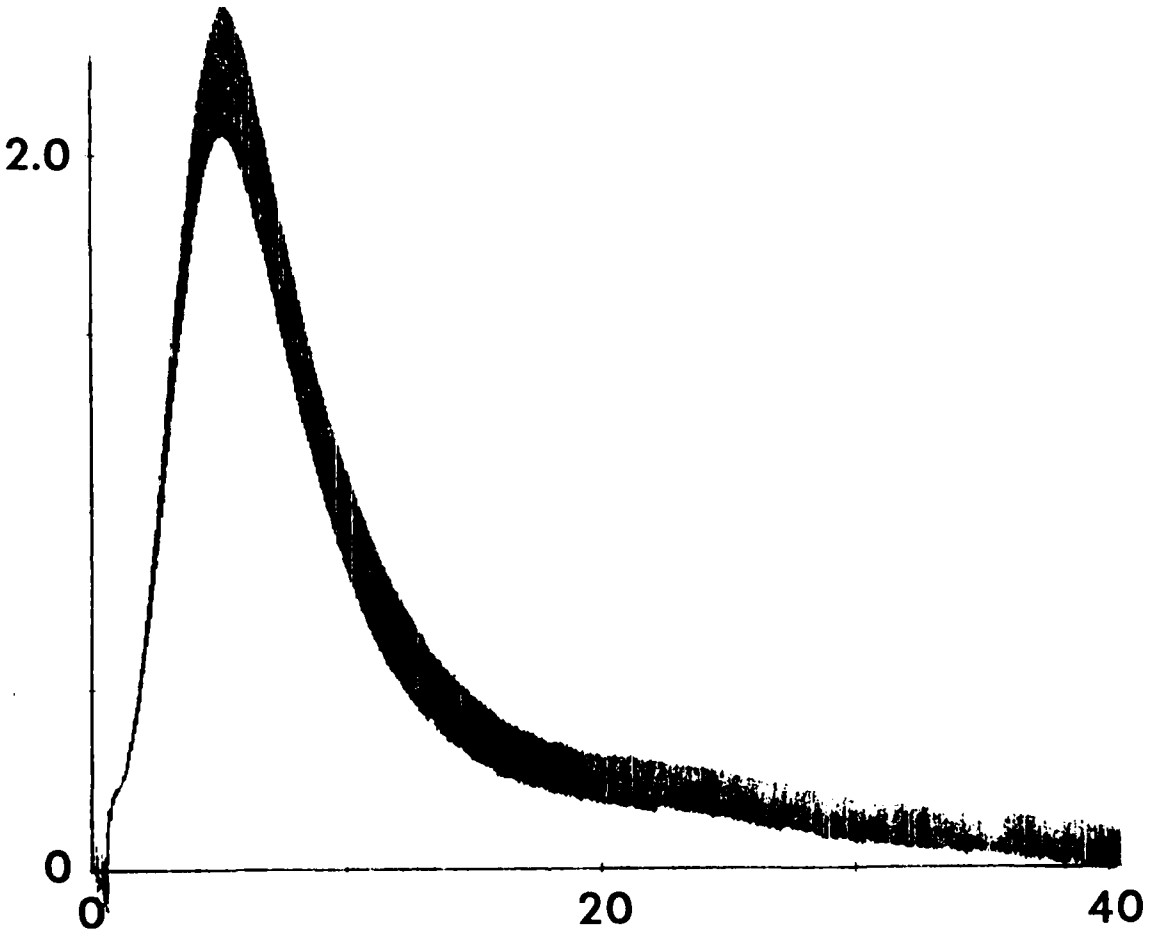

Figure 30. Plot derived from the data analysis programs developed for the LKB calorimetry system. The plot represents the mean of triplicate experiments with standard deviation. The Y axis is microcalories/sec/ml and the X axis is minutes.

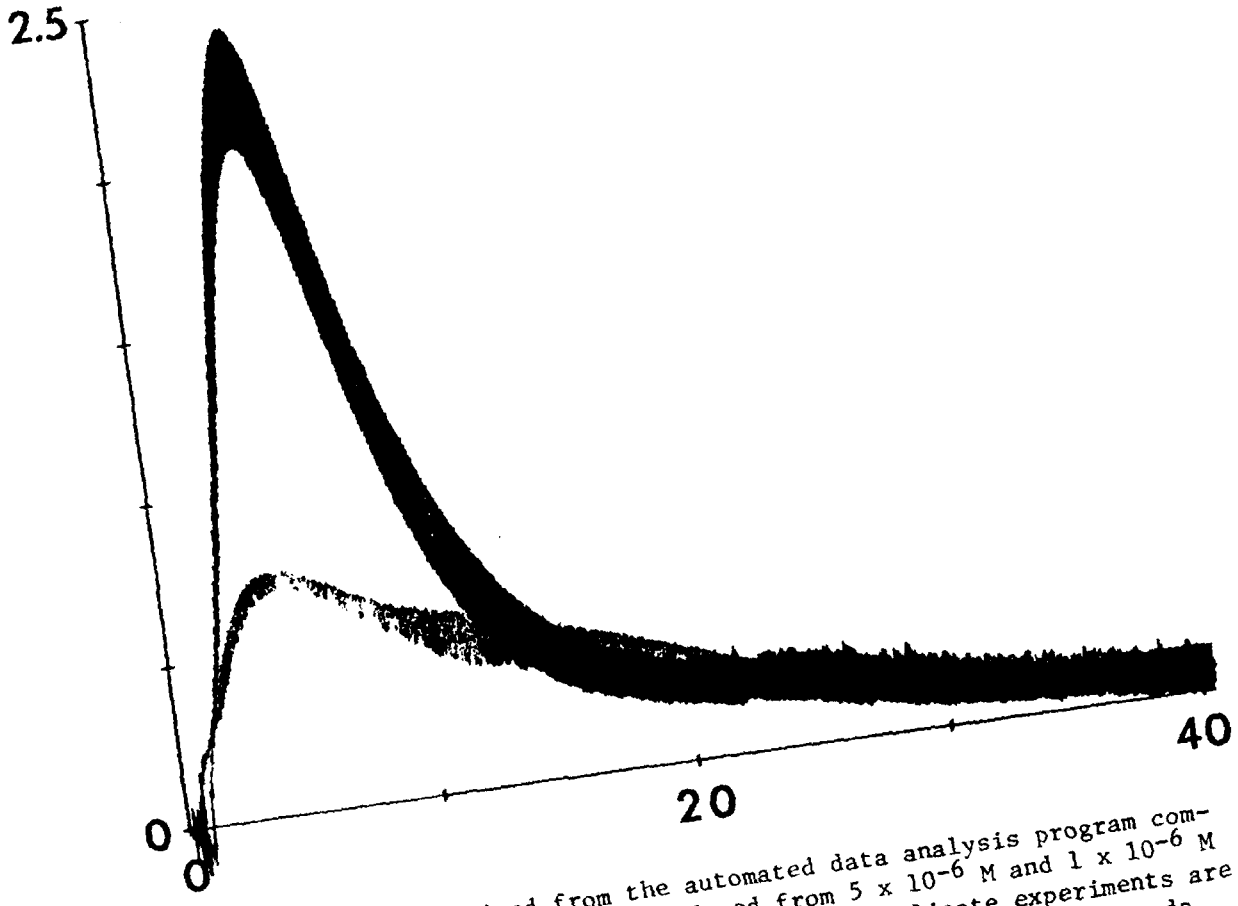

Figure 31. Plot derived from the automated data analysis program comparing thermograms produced from 5×10^{-6} M and 1×10^{-6} M glucose by 6×10^{7} bacteria/ml. Triplicate experiments are plotted as mean and standard deviation every 4.2 seconds. The Y axis is microcalories/sec/ml and the X axis is minutes.

Microcalorimetry was found to be a reasonably good technique for studying marine bacterial metabolism. Calorimetry appears to be a promising microbiological technique for several applications. Information from a specific thermogram yields a fingerprint of a specific organism which, although it may never be useful for identification of an unknown strain, is useful for monitoring a culture for changes due to contamination or mutation. A specific thermogram yields information on changes in growth rate and fermentation patterns as can be seen from the data presented on thermograms of the marine vibrios studied. Since thermograms such as those generated in the Tronac calorimeter reflect fermentation patterns, the technique would be an easy way to study parameters which affect fermentation balances. Specific peaks in the thermogram correspond to specific products and their area is proportional to the concentration of the metabolite excreted. In addition, the thermogram could be used to monitor the metabolism in large fermentors. The technique has been shown to be useful for studying the effects of toxicants, such as copper, on marine bacteria (Gordon and Millero, 1981).

Since both calorimetry and radiorespirometry were used in this study it may be of interest to discuss the pros and cons of these techniques.

Microcalorimetry is not as sensitive as

radiorespirometry. The best calorimeters can measure the metabolism of approximately 10^5 actively growing, aerobic bacteria per ml of solution. Another disadvantage of calorimetry is that under aerobic conditions no direct correlation of heat production with carbon fractionation between cells and end products can be made unless the enthalpy of growth is known. Only relative efficiencies can be measured unless the system of interest is calibrated with a technique such as respirometry. Calorimetry offers the advantage of giving continuous data and being easily automated. If the technique is sensitive enough for the application, calorimetry is probably the technique of choice for rate measurement especially if the conversion factor from measured heat production to respiration is also determined. The errors involved in calorimetric measurement appear to be greater than those of radiorespirometry in terms of integrated metabolism at micromolar substrate levels. This is probably due to the fact that the calorimeter is approaching its limit in sensitivity. The combination of calorimetry and respirometry allows one to measure the enthalpy of growth (H_a section 4.5.1) under aerobic conditions. This parameter may indeed be the best indication of the energy efficiency of microbial growth.

9.2 Appendix B- Programs for Analysis of Calorimetry Data with the Hewlett- Packard 9830 Computer System.

LISTING OF PROGRAMS FOR TRONAC CALORIMETRY

1) Transfer of data from paper tape to H.P. 9830

```
10 DIM A(100,20),Z$(3),C(10),B(10),P(2),S$(3)
20 N=P1=0
30 REM REVISION 11/7/79
40 REM ENTER DATA
50 HIGHCASE
60 DISP "LOAD TAPE,THEN TYPE GO                                    ";
70 REM
80 IF KEY(-1)#71 THEN 60
90 IF KEY(-1)#79 THEN 90
100 LOWCASE
110 REM
120 VDIM A(1000,2)
130 FOR I=1 TO 1000
140 ENTER (2,210)C,T
150 N=N+1
160 REM
170 REM
180 ARRAY A(N,1),Z,C
190 ARRAY A(N,2),Z,T
200 NEXT I
210 FORMAT 3X,F5.0,22X,F8.0
220 REM IF YOU CAME THROUGH THE LOOP YOU HAVE TOO MANY POINTS
230 BEEP
240 DISP " OVER 1000 POINTS TOO MANY FOR ME"
250 STOP
```

2) Enter data from magnetic tape

```
10 DIM A(100,20),B(10),C(10),Z$(3),P(2),S$(3)
20 REM enter stored data
30 P1=N=0
40 DISP "stored data file#";
50 INPUT F
60 LOAD  DATA F,A
70 REM find # points
80 FOR I=1 TO 100
90 FOR I2=1 TO 19 STEP 2
100 IF UND(A(I,I2)) THEN 160
110 N=N+1
120 NEXT I2
130 NEXT I
140 DISP "1000 points"
150 STOP
160 DISP N;"pionts"
170 END
```

3) Plot raw data

```
10 DIM A(100,20)
15 VDIM A(1000,2)
20 REM find max.,min.
30 REM initialize pointers
40 ARRAY A(1,1),B0
50 ARRAY A(1,2),B1
60 T1=B1
70 T0=B0
80 FOR I=2 TO N
90 DISP "calculating"
100 ARRAY A(I,1),Z
110 REM max,min counts
120 IF Z <= T0 THEN 160
130 T0=Z
140 GOTO 180
150 ARRAY A(I,1),Z
160 IF Z >= B0 THEN 180
170 B0=Z
180 REM find max,min volts
190 ARRAY A(I,2),Z
200 IF Z <= T1 THEN 230
220 GOTO 250
230 IF Z >= B1 THEN 250
240 B1=Z
250 NEXT I
260 REM scale
270 SCALE -10*N,110*N,B0-ABS(T0-B0)/10,T0+ABS(T0-B0)/10
280 XAXIS B0,1000,0,100*N
290 YAXIS 0,ABS(T0-B0),B0,T0
300 PLOT 0.2*N*100,B0-0.1*ABS(T0-B0),1
310 LABEL (*,2,2,0,1)
320 LABEL (*)"thousands  of seconds"
330 PEN
340 PLOT -5*N,0.3*(T0-B0)+B0,1
350 DEG
360 LABEL (*,2,2,90,1)"counts"
370 REM plot fast
380 FOR I=1 TO N
390 ARRAY A(I,1),Y
400 PLOT I*100,(T0-Y)+B0
410 NEXT I
420 PEN
430 P1=1
440 STOP
```

4) Edit data

```
5 DIM Z$(3)
10 REM editing
20 REM you must plot first to set up scales
30 PEN
40 I=0
50 IF P1#1 THEN 490
60 HIGHCASE
70 K=KEY(-1)
80 IF K=70 THEN 120
90 IF K=66 THEN 180
100 IF K=68 THEN 240
110 GOTO 70
120 REM move point forward
130 I=I+1
140 IF I>N THEN 70
150 ARRAY A(I,1),Z
160 PLOT I*100,(T0-Z)+B0
170 GOTO 70
180 REM plot backwards
190 I=I-1
200 IF I<1 THEN 70
210 ARRAY A(I,1),Z
220 PLOT I*100,(T0-Z)+B0
230 GOTO 70
240 REM delete point
250 REM deleted point=1/2(point-1)+1/2(point+1)
260 REM
270 DISP "delete are you sure";
280 INPUT Z$
290 Z$=Z$(1,1)
300 UCASE Z$
310 IF Z$#"y" THEN 70
320 REM see if there are points on both sides
330 IF I=1 THEN 400
340 IF I=N THEN 440
350 ARRAY A(I-1,1),X
360 ARRAY A(I+1,1),Y
370 Z=0.5*(X+Y)
380 ARRAY A(I+1),X,Z
390 GOTO 70
400 REM first point
410 ARRAY A(I+1,1),X
420 ARRAY A(I,1),Y,X
430 GOTO 70
440 REM last point
450 ARRAY A(I-1,1),X
460 ARRAY A(I,1),Y,X
470 GOTO 70
480 STOP
```

```
490 BEEP
500 DISP " you must plot first
510 END
```

5) Mark calibration heat

```
5 HIGHCASE
10 DIM C(10)
20 MAT C=ZER
30 I2=0
40 T=1000
50 DISP "F=FASTER, S=SLOWER"
60 WAIT 1000
65 DISP "PRESS T TO STOP"
66 WAIT 1000
70 DISP "  C MARKS CALIBRATION"
80 WAIT 1000
90 REM PLOT AND RECORD WHEN "C" IS PRESSED
100 FOR I=1 TO N
110 REM RECORD WHEN C WAS PUSHED
120 K=KEY(-1)
130 IF K#67 THEN 160
140 I2=I2+1
150 C(I2)=I
160 ARRAY A(I,1),Z
170 PLOT I*100,T0-Z+B0
180 WAIT T
190 IF K#70 THEN 210
200 T=INT(T/2)
210 IF K#83 THEN 225
220 T=2*T
225 IF K#84 THEN 230
226 ARRAY A(C(1),1),Z
227 ARRAY A(C(2),1),X
228 DISP "C(1),"Z" AND C(2),"X""
229 STOP
230 NEXT I
240 STOP
```

6) mark baseline level

```
5 HIGHCASE
10 DIM B(10)
20 MAT B=ZER
30 I2=0
40 T=1000
50 DISP "F= FASTER,S=SLOWER"
60 WAIT 1000
```

```
65 DISP " T TO STOP"
66 WAIT 1000
70 DISP "B MARKS BASELINE"
80 WAIT 1000
90 REM RECORD WHEN "B" IS PRESSED
100 FOR I=1 TO N
110 REM
120 K=KEY(-1)
130 IF K#66 THEN 160
140 I2=I2+1
150 B(I2)=I
160 ARRAY A(I,1),Z
170 PLOT I*100,T0-Z+B0
180 WAIT T
190 IF K#70 THEN 210
200 T=INT(T/2)
210 IF K#83 THEN 225
220 T=2*T
225 IF K#84 THEN 230
226 ARRAY A(B(1),1),Z
227 ARRAY A(B(2),1),X
228 DISP "B(1),"Z" AND B(2),"X""
229 STOP
230 NEXT I
240 STOP
```

7) Average calibration and baseline levels

```
10 I2=C1=B1=0
20 REM FIND AVE. C AND B
30 FOR I3=1 TO 9 STEP 2
40 FOR I=C(I3) TO C(I3+1)
45 IF I=0 THEN 100
50 ARRAY A(I,1),Z
60 C1=C1+Z
70 I2=I2+1
80 NEXT I
90 NEXT I3
100 C2=C1/I2
110 I2=0
120 FOR I3=1 TO 9 STEP 2
130 FOR I=B(I3) TO B(I3+1)
135 IF I=0 THEN 190
140 ARRAY A(I,1),Z
150 B1=B1+Z
160 I2=I2+1
170 NEXT I
180 NEXT I3
190 B2=B1/I2
200 DISP "DONE"
```

```
210 END
```

8) Enter constants

```
10 REM INPUT DATA
20 DISP "ENTER HTR V, HTR I";
30 INPUT H0,H1
40 DISP "ENTER MILLILITERS";
50 INPUT V
60 DISP "DO YOU WISH TO ENTER S";
70 INPUT S$
80 S$=S$(1,1)
90 UCASE S$
100 IF S$="N" THEN 140
110 IF S$="Y" THEN 120
120 DISP "ENTER S";
130 INPUT S
140 DISP "DONE"
150 END
```

9) Calculate scaling factor

```
10 REM CALCULATE SCALING FACTOR
20 S=H0*H1*10|6/(418.4*(B2-C2))/V
30 DISP "DONE"
40 END
```

10) Mark final plot

```
5 HIGHCASE
10 DIM P(2)
20 MAT P=ZER
30 I2=0
40 T=1000
50 DISP "F=FASTER,S=SLOWER"
60 WAIT 1000
70 DISP " P MARKS FINAL PLOT"
80 WAIT 1000
90 REM PLOT AND RECORD WHEN P IS PRESSED
100 FOR I=1 TO N
110 REM
120 K=KEY(-1)
130 IF K#80 THEN 160
140 I2=I2+1
150 P(I2)=I
160 ARRAY A(I,1),Z
170 PLOT I*100,T0-Z+B0
180 WAIT T
```

```
190 IF K#70 THEN 210
200 T=INT(T/2)
210 IF K#83 THEN 230
220 T=2*T
230 NEXT I
240 DISP "DONE"
250 END
```

11) Plot final plot

```
1 HIGHCASE
2 DISP "PUT A NEW PAPER ON AND TYPE GO";
3 WAIT 50000
4 IF KEY(-1)#71 THEN 4
5 IF KEY(-1)#79 THEN 5
10 DIM A(100,20)
20 VDIM A(1000,2)
21 F=((P(2)-P(1))*100)/3600
30 ARRAY A(P(1),1),B0
40 T0=B0
50 FOR I=(P(1)+1) TO P(2)
60 DISP "CALCULATING"
70 ARRAY A(I,1),Z
80 IF Z <= B0 THEN 110
90 IF Z >= T0 THEN 125
100 GOTO 129
110 B0=Z
120 GOTO 129
125 T0=Z
129 NEXT I
135 C=S*(T0-B0)
140 SCALE 0-0.1*F,F+0.1*F,0-0.1*C,C+0.1*C
150 XAXIS 0,1,0,F
160 YAXIS 0,10,0,C
170 ARRAY A(P(1),1),Y
180 PLOT (P(1)*100)/3600,S*(T0-Y),1
190 FOR I=P(1) TO P(2)
200 ARRAY A(I,1),Z
210 PLOT (100*I)/3600,S*(T0-Z),2
220 NEXT I
230 FOR I=0 TO F
240 LABEL (*,1.5,2,0,1)
250 PLOT I,0,1
260 CPLOT -1,-1
270 LABEL (*)I
280 NEXT I
285 DEG
290 FOR I=0 TO INT(C) STEP 10
300 PLOT 0,I,1
```

```
310 CPLOT -4,0
320 LABEL (*)I
330 NEXT I
340 PLOT 0.5*F,0-(0.25*C),1
350 CPLOT -2.5,0
360 LABEL (*,2,2,0,1)
370 LABEL (*)"HOURS"
380 PLOT 0-(0.2*F),0.5*C,1
390 CPLOT 0,-3.5
400 LABEL (*,2,2,90,1)
405 LABEL (*)"MICROCAL/SEC/ML"
410 DISP "DONE"
420 END
```

12) Output variables

```
10 PRINT "B0="B0"  T0="T0"  S="S""
20 PRINT
30 IF UND(B(2)) THEN 50
40 PRINT "B2="B2"  C2="C2""
50 PRINT
60 PRINT "P(1)="P(1)"  P(2)="P(2)""
65 PRINT
70 DISP "DONE"
80 END
```

13) Integrate

```
10 DIM A(100,20)
20 VDIM A(1000,2)
30 T=0
40 IF UND(P(1)) THEN 240
50 IF UND(B0) THEN 270
60 L=P(2)-P(1)
70 IF L/2#INT(L/2) THEN 90
80 GOTO 110
90 P(2)=P(2)-1
100 GOTO 60
110 ARRAY A(P(1),1),Z
120 ARRAY A(P(2),1),X
130 H=100
140 FOR I=(P(1)-1) TO (P(2)-1) STEP 2
150 ARRAY A(I,1),Q
160 ARRAY A((I+1),1),R
170 C=((H/3)*((4*(T0-Q))+(2*(T0-R))))
180 T=T+C
190 NEXT I
200 A=((H/3)*(Z-X))+T
205 IF UND(V) THEN 295
```

```
210 A=A*S*1E-06*V
220 PRINT "THERMOGRAM AREA="A" CALORIES"
230 STOP
240 BEEP
250 DISP "FIRST MARK FINAL PLOT"
260 STOP
270 BEEP
280 DISP "FIRST PLOT FINAL PLOT"
290 STOP
295 DISP "ENTER MILLILITERS"
300 INPUT V
310 GOTO 210
320 END
```

LKB CALORIMETRY PROGRAMS

1) Transfer data from paper tape

```
10 DIM A(100,20),Z$(3),C(10),B(10),P(2),S$(3),D$(3)
20 N=P1=0
30 REM REVISION 11/7/79
40 REM ENTER DATA
50 HIGHCASE
60 DISP "LOAD TAPE,THEN TYPE GO
70 REM
80 IF KEY(-1)#71 THEN 60
90 IF KEY(-1)#79 THEN 90
100 LOWCASE
110 REM
120 VDIM A(2000,1)
130 FOR I=1 TO 2000
140 ENTER (2,200)C,T
150 N=N+1
160 REM
170 REM
180 ARRAY A(N,1),Z,C
190 NEXT I
200 FORMAT F7.0,1X,F1.0
210 REM IF YOU CAME THROUGH THE LOOP YOU HAVE TOO MANY POINTS
220 BEEP
230 DISP " OVER 2000 POINTS TOO MANY FOR ME"
240 STOP
```

2) Enter data stored on magnetic tape

```
10 DIM A(100,20),B(10),C(10),Z$(3),P(2),S$(3),D$(3)
20 REM enter stored data
30 P1=N=0
40 DISP "stored data file#";
50 INPUT F
60 LOAD  DATA F,A
70 REM find # points
80 FOR I=1 TO 100
90 FOR I2=1 TO 19 STEP 2
100 IF UND(A(I,I2)) THEN 155
110 N=N+1
120 NEXT I2
130 NEXT I
140 DISP "2000 points"
150 STOP
155 N=2*N
160 DISP N;"points"
170 END
```

3) plot raw data

```
10 DIM A(100,20)
20 VDIM A(2000,1)
30 REM find max.,min.
40 REM initialize pointers
50 ARRAY A(1,1),B0
60 B0=B0/1000
70 T0=B0
80 FOR I=2 TO N-1
90 DISP "calculating"
100 ARRAY A(I,1),Z
110 Z=Z/1000
120 REM max,min v0ltage
130 IF Z <= T0 THEN 180
140 T0=Z
150 GOTO 200
160 ARRAY A(I,1),Z
170 Z=Z/1000
180 IF Z >= B0 THEN 200
190 B0=Z
200 NEXT I
210 REM scale
220 A=(T0-B0)
230 SCALE 0-(0.42*N),4.2*N+(0.42*N),(B0)-(0.2*A),(T0)+(0.2*A)
240 XAXIS (B0),42,0,4.2*N
250 YAXIS 0,(A)/5,(B0),(T0)
260 PLOT 0.3*N*4.2,(B0)-(0.1*(A)),1
270 LABEL (*,2,2,0,1)
280 LABEL (*)"42 seconds"
290 PEN
300 PLOT 0-(0.21*N),0.3*(A)+(B0),1
310 DEG
320 LABEL (*,2,2,90,1)"millivolts"
330 REM plot fast
340 FOR I=1 TO N-1
350 ARRAY A(I,1),Y
360 Y=Y/1000
370 PLOT I*4.2,(Y)
380 NEXT I
390 PEN
400 P1=1
410 STOP
```

4) Edit data

```
10 DIM Z$(3)
20 REM editing
30 REM you must plot first to set up scales
40 PEN
50 I=0
60 IF P1#1 THEN 520
70 HIGHCASE
80 K=KEY(-1)
90 IF K=70 THEN 130
100 IF K=66 THEN 200
110 IF K=68 THEN 270
120 GOTO 80
130 REM move point forward
140 I=I+1
150 IF I>N THEN 80
160 ARRAY A(I,1),Z
170 Z=Z/1000
180 PLOT I*4.2,(Z)
190 GOTO 80
200 REM plot backwards
210 I=I-1
220 IF I<1 THEN 80
230 ARRAY A(I,1),Z
240 Z=Z/1000
250 PLOT I*4.2,(Z)
260 GOTO 80
270 REM delete point
280 REM deleted point=1/2(point-1)+1/2(point+1)
290 REM
300 DISP "delete are you sure";
310 INPUT Z$
320 Z$=Z$(1,1)
330 UCASE Z$
340 IF Z$#"Y" THEN 80
350 REM see if there are points on both sides
360 IF I=1 THEN 430
370 IF I=N THEN 470
380 ARRAY A(I-1,1),X
390 ARRAY A(I+1,1),Y
400 Z=0.5*(X+Y)
410 ARRAY A(I,1),X,Z
420 GOTO 80
430 REM first point
440 ARRAY A(I+1,1),X
450 ARRAY A(I,1),Y,X
460 GOTO 80
470 REM last point
480 ARRAY A(I-1,1),X
490 ARRAY A(I,1),Y,X
```

";

```
500 GOTO 80
510 STOP
520 BEEP
530 DISP " you must plot first
540 END
```

5) Calculate scaling factor

```
10 REM CALCULATE SCALING FACTOR
20 DISP "FROM AREA OR HEIGHT,1 OR 2";
30 INPUT B
40 IF B=1 THEN 80
50 S=-((M|2*50.3*1E+06)/4.184/(C2-B2)/V)
60 DISP "DONE"
70 STOP
80 IF UND(A) THEN 120
90 S=(M|2*50.3*G*1E+06)/(4.184*A*V)
100 PRINT "S="S""
110 END
120 DISP "ENTER AREA IN MILLIVOLTS";
130 INPUT A
140 GOTO 90
150 END
```

6) Mark final plot

```
10 HIGHCASE
20 DIM P(2)
30 MAT P=ZER
40 I2=0
50 T=1000
60 DISP "F=FASTER,S=SLOWER"
70 WAIT 1000
80 DISP " P MARKS FINAL PLOT"
90 WAIT 1000
100 REM PLOT AND RECORD WHEN P IS PRESSED
110 FOR I=1 TO N
120 REM
130 K=KEY(-1)
140 IF K#80 THEN 170
150 I2=I2+1
160 P(I2)=I
170 ARRAY A(I,1),Z
180 Z=Z/1000
190 PLOT I*4.2,(Z)
200 WAIT T
210 IF K#70 THEN 230
220 T=INT(T/2)
230 IF K#83 THEN 250
```

```
240 T=2*T
250 NEXT I
260 DISP "DONE"
270 END
```

7) Plot final plot

```
10 DIM D$(3),A(100,20)
15 VDIM A(2000,1)
20 DISP "do you want baseline correction";
30 INPUT D$
40 UCASE D$
50 D$=D$(1,1)
60 IF D$="Y" THEN 670
70 HIGHCASE
80 DISP "PUT A NEW PAPER ON AND TYPE GO";
90 WAIT 50000
100 IF KEY(-1)#71 THEN 100
110 IF KEY(-1)#79 THEN 110
120 DIM A(100,20)
130 VDIM A(2000,1)
140 F=((P(2)-P(1))*4.2)/600
150 ARRAY A(P(1),1),B0
160 B0=B0/1000
170 T0=B0
180 FOR I=(P(1)+1) TO P(2)
190 DISP "CALCULATING"
200 ARRAY A(I,1),Z
210 Z=Z/1000
220 IF D$#"Y" THEN 240
230 Z=Z-B*(I-P(1))
240 IF Z <= B0 THEN 270
250 IF Z >= T0 THEN 290
260 GOTO 300
270 B0=Z
280 GOTO 300
290 T0=Z
300 NEXT I
310 C=(S*(T0-B0))
320 SCALE 0-0.1*F,F+0.1*F,0-0.1*C,C+0.1*C
330 XAXIS 0,1,0,F
340 YAXIS 0,0.5,0,(C)
350 ARRAY A(P(1),1),Y
360 Y=Y/1000
370 PLOT 0,(S*((Y)-(B0))),1
380 FOR I=P(1) TO P(2)
390 ARRAY A(I,1),Z
400 Z=Z/1000
410 IF D$#"Y" THEN 430
420 Z=Z-B*(I-P(1))
```

```
430 PLOT ((I-P(1))*4.2)/600,(S*((Z)-(B0))),2
440 NEXT I
450 FOR I=0 TO F
460 LABEL (*,1.5,2,0,1)
470 PLOT I,0,1
480 CPLOT -1,-1
490 LABEL (*)I
500 NEXT I
510 DEG
520 FOR I=0 TO C STEP 0.5
530 PLOT 0,I,1
540 CPLOT -4,0
550 LABEL (*)I
560 NEXT I
570 PLOT 0.5*F,0-(0.07*C),1
580 CPLOT -0.5,0
590 LABEL (*,2,2,0,1)
600 LABEL (*)"MINUTES  *10"
610 PLOT 0-(0.05*F),0.5*C,1
620 CPLOT 0,-3.5
630 LABEL (*,2,2,90,1)
640 LABEL (*)"MICROCAL/SEC/ML"
650 DISP "DONE"
660 END
670 ARRAY A(P(1),1),Z
680 ARRAY A(P(2),1),X
690 Z=Z/1000
700 X=X/1000
710 B=(X-Z)/(P(2)-P(1))
720 GOTO 70
730 END
```

8) Output variables

```
10 FORMAT "B0=",F8.3," T0=",F8.3,""
20 WRITE (15,10)B0,T0
30 FORMAT "P(1)=",F6.0,"  P(2)=",F6.0,"  N=",F6.0,""
40 WRITE (15,30)P(1),P(2),N
50 FORMAT "S=",F8.4,""
60 WRITE (15,50)S
70 PRINT
80 PRINT
90 PRINT "CULTURE CONDITION:"
100 PRINT "GLUCOSE C:"
110 PRINT "MEDIA:"
120 DISP "DONE"
130 END
```

9) Input constants

```
10 REM INPUT DATA
20 DISP "ENTER AMPS CALIB.     ";
30 INPUT M
31 DISP "ENTER TIME CALIB.";
32 INPUT G
40 DISP "ENTER MILLILITERS";
50 INPUT V
60 DISP "DO YOU WISH TO ENTER S";
70 INPUT S$
80 S$=S$(1,1)
90 UCASE S$
100 IF S$="N" THEN 140
110 IF S$="Y" THEN 120
120 DISP "ENTER S";
130 INPUT S
140 DISP "DONE"
150 END
```

10) Integrate

```
10 DIM A(100,20)
20 VDIM A(2000,1)
30 DISP "MILLIVOLTS OR CALORIES,1 OR 2";
40 INPUT J
50 Y=T=A=0
60 IF UND(P(1)) THEN 440
70 L=P(2)-P(1)
80 IF L/2#INT(L/2) THEN 100
90 GOTO 120
100 P(2)=P(2)-1
110 GOTO 70
120 ARRAY A(P(1),1),Z
130 ZZ/1000
140 ARRAY A(P(2),1),X
150 X=X/1000
160 H=4.2
170 FOR I=(P(1)-1) TO(P(2)-1) STEP 2
180 DISP ""I"            BUSY              "
190 ARRAY A(I,1),Q
200 Q=Q/1000
210 ARRAY A((I+1),1),R
220 R=R/1000
230 C=((H/3)*((4*(Q-B0))+(2*(R-B0))))
240 T=T+C
250 NEXT I
260 A=((H/3)*(Z-X))+T
270 REM CORRECT BASELINE
280 Y=ABS((Z-X)*L*H)/2
```

```
290 DISP "RECT OUT? 0=NO,1=YES";
300 INPUT W
310 IF W=0 THEN 360
320 IF Z>X THEN 350
330 Y=Y+((Z-B0)*L*H)
340 GOTO 360
350 Y=Y+((X-B0)*L*H)
360 A=A-Y
370 IF UND(V) THEN 470
380 IF J=1 THEN 420
390 A=A*S*1E-06*V
400 WRITE (15,500)A*1000
410 GOTO 430
420 WRITE (15,510)A
430 STOP
440 BEEP
450 DISP "FIRST MARK FINAL PLOT"
460 STOP
470 DISP "ENTER MILLILITERS";
480 INPUT V
490 GOTO 390
500 FORMAT "THERMOGRAM AREA=",F9.3," MILLICALORIES"
510 FORMAT "THERMOGRAM AREA=",F15.3," MILLIVOLTS"
520 END
```

11) Coplot thermograms

```
10 DIM A(100,20),D$(3)
20 DISP "ENTER XMIN,XMAX,INCR.";
30 INPUT X1,X2,X3
40 DISP " ENTER YMIN,YMAX,INCR.";
50 INPUT Y1,Y2,Y3
60 L=X2-X1
70 M=Y2-Y1
80 SCALE X1-0.1*L,X2+0.1*L,Y1-0.1*M,Y2+0.1*M
90 XAXIS 0,X3,X1,X2
100 YAXIS 0,Y3,Y1,Y2
110 FOR I=0 TO X2 STEP X3
120 LABEL (*,1.5,2,0,1)
130 PLOT I,0,1
140 CPLOT -1,-1
150 LABEL (*)I
160 NEXT I
170 DEG
180 FOR I=0 TO Y2 STEP Y3
190 PLOT 0,I,1
200 CPLOT -4,0
210 LABEL (*)I
220 NEXT I
230 PLOT 0.5*L,0-(0.5*M),1
```

```
240 CPLOT -0.5,0
250 LABEL (*,2,2,0,1)
260 LABEL (*)"* 10 MINUTES"
270 PLOT 0-(0.05*L),0.5*M,1
280 CPLOT 0,-3
290 LABEL (*,2,2,90,1)
300 LABEL (*)"MICROCAL/SEC/ML"
310 DISP "IS DATA ALREADY IN MEMORY,0=NO,1=YES";
320 INPUT D1
330 IF D1=1 THEN 360
340 DISP "ENTER DATA FILE#";
350 INPUT F
360 DISP "ENTER SCALING FACTOR";
370 INPUT S
380 DISP "ENTER START AND END POINT";
390 INPUT B1,E1
400 DISP "ENTER B(0)";
410 INPUT T
420 DISP "DO YOU WANT BASELINE CORRECTION";
430 INPUT D$
440 D$=D$(1,1)
450 UCASE D$
460 IF D1=1 THEN 490
470 VDIM A(2000,1)
480 LOAD  DATA F,A
490 IF D$="Y" THEN 610
500 PLOT 0,0,1
510 FOR I=B1 TO E1
520 ARRAY A(I,1),Z
530 Z=Z/1000
540 IF D$#"Y" THEN 560
550 Z=Z-(B*(I-B1))
560 PLOT (I-B1)*4.2/600,(S*(Z-T)),2
570 NEXT I
580 PEN
590 DISP "FOR ANOTHER PLOT,CONT 310 EXEC.";
600 STOP
610 ARRAY A(B1,1),Z
620 ARRAY A(E1,1),X
630 Z=Z/1000
640 X=X/1000
650 B=(X-Z)/(E1-B1)
660 GOTO 500
670 END
```

12) Edit data files for coplotting with errors

```
10 REM LKB DATA EDITING ROUTINE
20 REM STORES SELECTED POINTS ON TAPE
30 DIM A(100,20),B(3,200)
40 MAT B=ZER
50 DISP "ENTER FILE FOR EDITING";
60 INPUT D
70 LOAD  DATA D,A
80 DISP "ENTER P(1) AND P(2)";
90 INPUT P1,P2
100 P1=P1-1
110 FOR I=1 TO 3
120 FOR J=1 TO 200
130 P1=P1+1
140 VDIM A(2000,1)
150 ARRAY A(P1,1),Z
160 B(I,J)=Z
170 IF P1=P2 THEN 200
180 NEXT J
190 NEXT I
200 DISP "ENTER FILE TO STORE EDITED DATA";
210 INPUT F
220 STORE  DATA F,B
230 DISP "DONE";
240 END
```

13) coplot thermograms with errors

```
10 REM AVERAGE DATA,CALCULATE SIGMA AND PLOT
20 DIM A(200,3),B(200,3),C(200,3),D(200,3)
30 DISP "3 DATA FILES MAX. HOW MANY?";
40 INPUT M
50 DISP " HOW MANY POINTS PER FILE";
60 INPUT N
70 IF M=2 THEN 360
80 DISP "ENTER DATA FILES";
90 INPUT F1,F2,F3
100 LOAD  DATA F1,A
110 LOAD  DATA F2,B
120 LOAD  DATA F3,C
130 REM NORMALIZE BASELINES
140 VDIM A(600,1)
150 ARRAY A(1,1),Z
160 FOR I=1 TO N
170 ARRAY A(I,1),X
180 X=X-Z
190 ARRAY A(I,1),Y,X
200 NEXT I
```

```
210 VDIM B(600,1)
220 ARRAY B(1,1),Z
230 FOR I=1 TO N
240 ARRAY B(I,1),X
250 X=X-Z
260 ARRAY B(I,1),Y,X
270 NEXT I
280 VDIM C(600,1)
290 ARRAY C(1,1),Z
300 FOR I=1 TO N
310 ARRAY C(I,1),X
320 X=X-Z
330 ARRAY C(I,1),Y,X
340 NEXT I
350 GOTO 550
360 DISP "ENTER DATA FILES";
370 INPUT F1,F2
380 LOAD  DATA F1,A
390 LOAD  DATA F2,B
400 VDIM A(600,1)
410 ARRAY A(1,1),Z
420 FOR I=1 TO N
430 ARRAY A(I,1),X
440 X=X-Z
450 ARRAY A(I,1),Y,X
460 NEXT I
470 VDIM B(600,1)
480 ARRAY B(1,1),Z
490 FOR I=1 TO N
500 ARRAY B(I,1),X
510 X=X-Z
520 ARRAY B(I,1),Y,X
530 NEXT I
540 GOTO 920
550 REM ADD MATRICES
560 MAT D=A+B
570 MAT D=D+C
580 REM AVERAGE MATRICES
590 MAT D=(1/3)*D
600 REM CALCULATE SIGMA FOR EACH POINT
610 MAT A=A-D
620 MAT B=B-D
630 MAT C=C-D
640 VDIM A(600,1)
650 FOR I=1 TO 600
660 ARRAY A(I,1),Z
670 Z=Z*Z
680 ARRAY A(I,1),X,Z
690 NEXT I
700 VDIM B(600,1)
710 FOR I=1 TO 600
```

```
720 ARRAY B(I,1),Z
730 Z=Z*Z
740 ARRAY B(I,1),X,Z
750 NEXT I
760 VDIM C(600,1)
770 FOR I=1 TO 600
780 ARRAY C(I,1),Z
790 Z=Z*Z
800 ARRAY C(I,1),X,Z
810 NEXT I
820 MAT A=A+B
830 MAT A=A+C
840 MAT A=(1/M)*A
850 VDIM A(600,1)
860 FOR I=1 TO 600
870 ARRAY A(I,1),Z
880 Z=SQR(Z)
890 ARRAY A(I,1),X,Z
900 NEXT I
910 GOTO 1170
920 REM CALCULATE FOR TWO DATA SETS
930 MAT D=A+B
940 MAT D=(1/2)*D
950 MAT A=A-D
960 MAT B=B-D
970 VDIM A(600,1)
980 FOR I=1 TO 600
990 ARRAY A(I,1),Z
1000 Z=Z*Z
1010 ARRAY A(I,1),X,Z
1020 NEXT I
1030 VDIM B(600,1)
1040 FOR I=1 TO 600
1050 ARRAY B(I,1),Z
1060 Z=Z*Z
1070 ARRAY B(I,1),X,Z
1080 NEXT I
1090 MAT A=A+B
1100 MAT A=(1/M)*A
1110 VDIM A(600,1)
1120 FOR I=1 TO 600
1130 ARRAY A(I,1),Z
1140 Z=SQR(Z)
1150 ARRAY A(I,1),X,Z
1160 NEXT I
1170 REM NOW PLOT AVERAGE AND SIGMA FIOR EACH POINT
1180 DISP " DO YOU WISH TO SPECIFY SCALE ,ENTER 0 FOR NO, 1 FOR YES";
1190 FOR I=1 TO 25
1200 SCROLLL 300
1210 NEXT I
1220 INPUT Q
```

```
1221 IF Q=1 THEN 1600
1230 REM CALCULATE MIN. AND MAX. VOLTAGE
1240 VDIM D(600,1)
1250 ARRAY D(1,1),Z
1260 B0=T0=Z/1000
1270 FOR I=1 TO N
1280 DISP "CALCULATING"
1290 ARRAY D(I,1),Z
1300 Z=Z/1000
1310 IF Z <= B0 THEN 1340
1320 IF Z >= T0 THEN 1360
1330 GOTO 1370
1340 B0=Z
1350 GOTO 1370
1360 T0=Z
1370 NEXT I
1380 DISP "ENTER AVERAGE SCALING FACTOR";
1390 INPUT S
1395 IF Q=1 THEN 1650
1400 REM PLOT DATA AND ERROR BARS
1410 F=N*0.007
1420 C=S*(T0-B0)
1430 SCALE 0-0.1*F,F+0.1*F,0-0.1*C,C+0.1*C
1440 XAXIS 0,1,0,F
1450 YAXIS 0,0.5,0,C
1460 PLOT 0,0,1
1470 FOR I=1 TO N
1480 VDIM D(600,1)
1490 ARRAY D(I,1),Z
1500 Z=Z/1000
1510 PLOT I*0.007,(S*(Z)),2
1520 VDIM A(600,1)
1530 ARRAY A(I,1),X
1540 X=X/1000
1550 PLOT I*0.007,(S*(Z-X)),2
1560 PLOT I*0.007,(S*(Z+X)),2
1570 PLOT I*0.007,(S*(Z)),2
1580 NEXT I
1590 END
1600 DISP "ENTER XMAX AND INCREMENT";
1610 INPUT X1,X2
1620 DISP "ENTER Y MAX. AND INCREMENT";
1630 INPUT Y1,Y2
1640 GOTO 1380
1650 SCALE 0-0.1*X1,X1+0.1*X1,0-0.1*Y1,Y1+0.1*Y1
1660 XAXIS 0,X2,0,X1
1670 YAXIS 0,Y2,0,Y1
1680 GOTO 1460
1690 END
```

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VITA

Andrew Sirkosky Gordon was born in Washington, D.C. on May 23, 1954. His parents are Dr. Robert S. Gordon Jr. and Elizabeth Brown Gordon. He received his elementary education at Kensington Elementary School, Kensington, MD and his secondary education at Albert Einstein High School in Kensington, MD and the Woodstock Country School in South Woodstock, VT. He recieved his B.A. in oceanography from Cathlolic University in Washington, D.C. in 1976. He entered the University of Miami graduate school in 1976 and was granted a Ph.D. in microbiology in May 1982.

Permanent address: 5858 S.W. 81st street, Miami, FL 33143

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4. TITLE (and Subtitle) The Effect of Inorganic Particles on Metabolism by Marine Bacteria	5. TYPE OF REPORT & PERIOD COVERED 6. PERFORMING ORG. REPORT NUMBER 82002
7. AUTHOR(s) Andrew S. Gordon	8. CONTRACT OR GRANT NUMBER(s) N00014-80-C-0042
9. PERFORMING ORGANIZATION NAME AND ADDRESS Rosenstiel School of Mar. & Atm. Science 4600 Rickenbacker Cswy. Miami, FL 33149	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS NR-083-060
11. CONTROLLING OFFICE NAME AND ADDRESS ONR-420	12. REPORT DATE May 1982 13. NUMBER OF PAGES 141
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office) same	15. SECURITY CLASS. (of this report) unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Inorganic particles, Marine bacteria, Hydroxyapatite

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Measurements were made of adsorption of organic nutrients and bacteria onto the surface of inorganic particles. The effect of the particles on the metabolism of bacteria attached to surfaces and free in solution was determined.

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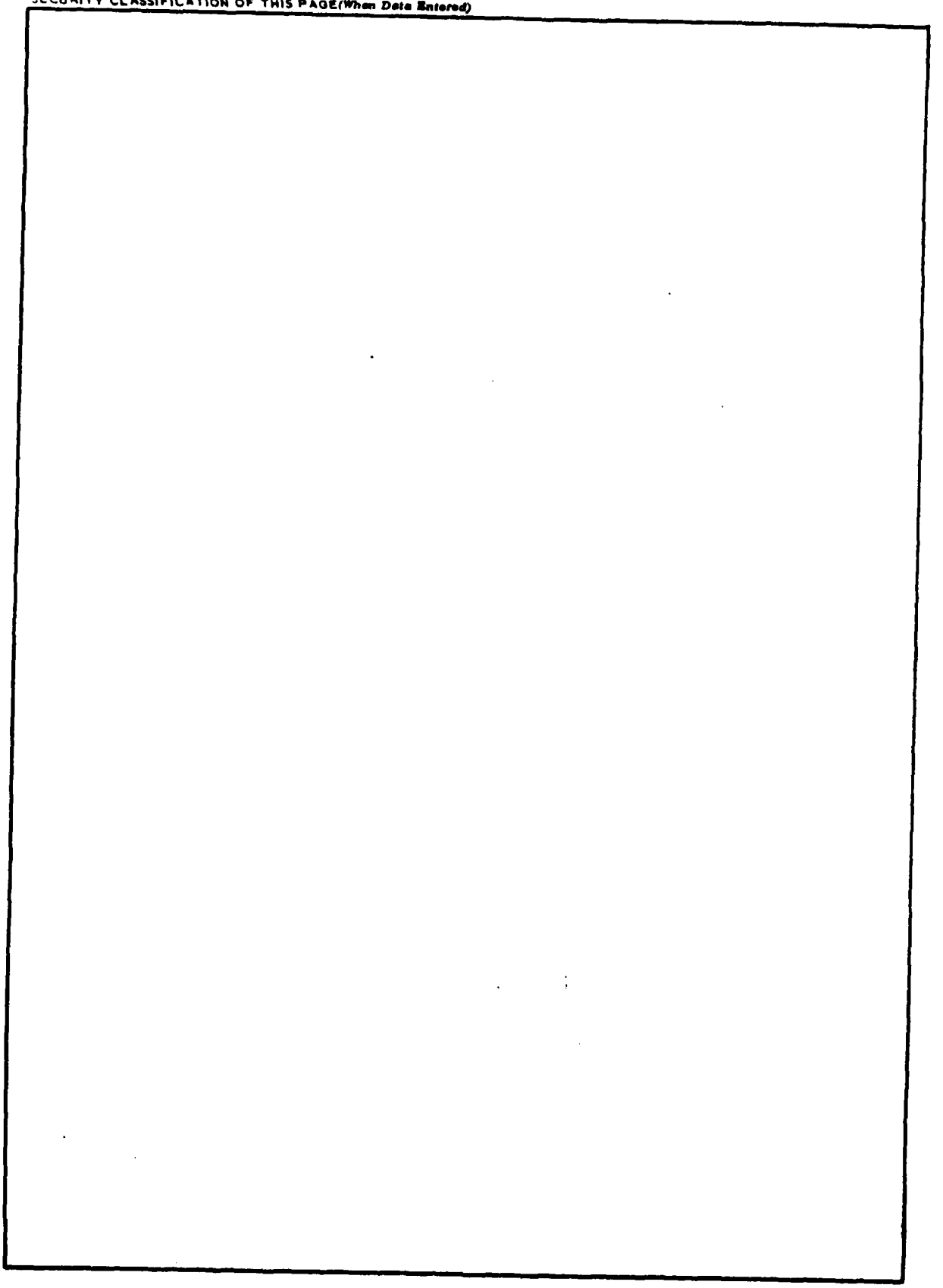

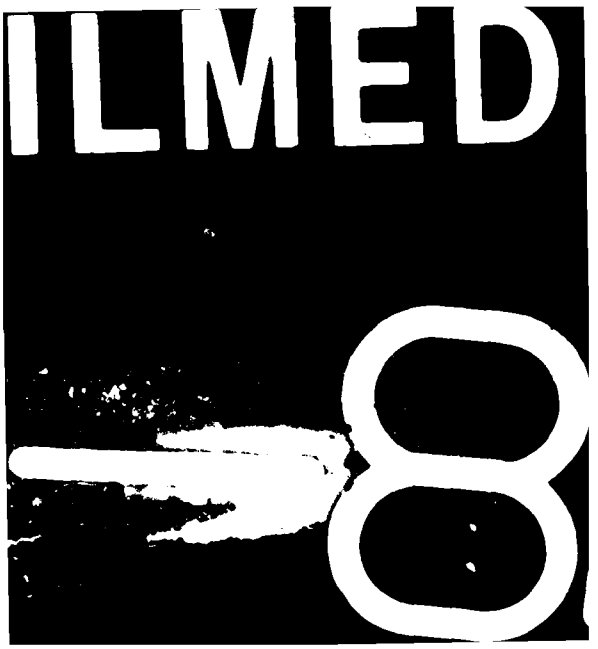
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